

nature

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The unloved treaty

In two months time those countries which have ratified the Non-Proliferation Treaty (NPT) will be reviewing, under UN auspices in Geneva, the first five years of the treaty. There is no doubt that in 1970 the hopes (at least of the non-nuclear countries) were that by 1975 the NPT would be a solid foundation on which to build further arms-control measures, aimed at progressively steering the world away from the contemplation of nuclear solutions to political problems. Certainly in the past five years nobody has fired a nuclear weapon in anger, though the NPT could hardly claim much credit for that. On the other hand the prospects for a nuclear-free future are no better than they were in 1970 and the NPT foundations have been neglected—indeed show signs of crumbling.

The treaty bans the development of nuclear explosives of any kind in non-weapon states in exchange for promises that the benefits of peaceful applications will be extended to those states. There are some notable absences from the list of ratifiers; a few non-ratifiers have signed but will not ratify for fairly specific reasons, but the following countries *inter alia* have not even declared interest in the treaty at all: Argentina, Brazil, Chile, China, France, India, Israel, Pakistan, Portugal, South Africa and Spain. The weight of the three original nuclear powers which used to reassure other countries that treaties were worth signing is now counterbalanced by the weight of three other nuclear powers who want nothing to do with this treaty.

The reasons for the failure of the NPT to catch on are all too obvious—it is asking for major concessions from the 'have-nots' in exchange for minimal concessions from the 'haves', and it is a trap for have-nots who sign and find that their unfriendly neighbours do not follow suit. Much has been written on the extent to which vertical proliferation, the continued refinement of the nuclear nations' arsenal, causes offence to non-nuclear powers. There has been remarkably little public discussion of the real reason for the diffidence of most potentially nuclear states towards the NPT—the fear of unbalanced horizontal proliferation which, in the absence of simultaneous accession by all states, cannot be ruled out.

It is widely believed that the NPT contains assurances to non-nuclear states in the event of nuclear attacks. This is not so. A resolution of the UN Security Council, which appeared when the NPT was first opened for signature, replaces any binding commitment by a general welcome for the intention of the UK, the USA and the Soviet Union to act "in accordance with the Charter" were any non-nuclear-weapon state, party to the NPT, to be the victim of an act, or the object of a threat, of nuclear aggression. The catch is that the Security Council with all its possibility for veto would be the vehicle for action. Small wonder that countries which have refused to sign the NPT abstained on this resolution also.

Finally, the NPT promised that benefits from peaceful nuclear explosives would be made available "through an international body". Negotiations were to start "as soon as possible". Back in 1968 when the treaty was drafted, the Plowshare programme in the United States was languishing. In major excavation projects, for instance, costs for even chemical-explosive techniques were double those for conventional techniques. Since then, however, things have changed dramatically. The factor of two in cost is now a factor of much less than one; in the future the same will be true of nuclear explosives.

In the Soviet Union, in an entirely different political and physical environment, the cross-over point must have been reached much earlier, for nuclear explosive projects burgeoned in 1970 and now run at the rate of 10 a year. Thus the hopes of many in 1968 that peaceful applications would never be economic and thus might not obstruct arms control discussions have been proved unfounded.

So where is the international body? It is now five years since the treaty came into force and the International Atomic Energy Agency (IAEA) has yet to establish any body to consider the political and legal aspects of an international service. In the meantime, the Soviet Union, it is said, took it upon herself to offer India nuclear explosives for peaceful purposes but was rebuffed. Some minds had better be made up soon on the establishment of a body; the next bilateral deal may be accepted.

The IAEA has not been entirely inactive. Technical panels have exchanged a substantial amount of information and non-nuclear countries have been party to all this. But the political nettle has yet to be grasped.

The reasons are a combination of bureaucracy, politics and genuine concern for safety. There is a resistance in Vienna to the accumulation of yet more duties for a secretariat already fully extended. There is concern that the IAEA might include only NPT-ratifiers amongst beneficiaries of nuclear technology. And there is a fear that the apparent legitimisation of nuclear explosive transfers will lead to the even wider dissemination of potential weapons material, and will be an encouragement to even more countries to think nuclear. This last is a fairly weak objection now that events have overtaken hopes that peaceful explosions might not prove viable. The IAEA itself these days puts out fairly enthusiastic publicity for nuclear explosives in its technical reports. There is, however, some substance to fears that the service might be tied to the NPT. Any restrictions of this sort, although at first sight a means of bolstering up the treaty, would in the long run diminish IAEA initiatives by driving non-ratifiers into more implacable opposition.

On many grounds the NPT has not lived up to expectations and could even be seen as creating opposing nuclear camps. Will anyone have the courage in May to suggest the treaty be scrapped and new approaches tried?



AS a country Britain is living beyond its means, and it is surely no more than good sense to consider ways in which we can meet more of our needs from our own resources, among which agricultural resources figure prominently. In the past 100 years or so the latter have been evaluated against a background of abundant world supplies of food, available freely except for short periods of war and post-war restriction, and to be purchased cheaply in relation to the price of the industrial goods we had to offer in exchange. We have rarely had an explicitly formulated agricultural policy, but the *de facto* policy that has grown up has resulted from the impact of these world forces.

The world situation has changed, dramatically and to our disadvantage. Although agricultural production has increased enormously over the past quarter century, the increase has been taken up by population growth and by the increasing sophistication of the diets of affluent countries. Thus, now that the basic resources on which farm production depends—land, fertilisers and energy—have become scarce and costly, we have no margin to protect us against shortage; prices have risen greatly in rich countries, and poor countries face widespread hunger. In this context we should reappraise our agricultural assets, with a view to meeting from them more of our needs.

In Britain we enjoy a generous, not to say extravagant, diet, including a high proportion of animal products. The livestock industry has come to depend very heavily on concentrate feeding, and home feed production is augmented by massive imports of coarse grains and protein feedstuffs. In present circumstances this is a profligate way of feeding ourselves, and we know that we could devise a palatable and nutritionally adequate diet at much less expense in real resources, and with much less dependence on imports. A reduction in the heavy consumption of animal products would make a major contribution to Britain's balance of payments, and would help ease the world food situation.

The corresponding changes in the production pattern of British agriculture would require extensive planning, and would take some years to implement in full. It is, therefore, particularly important that we should start planning without delay. Planning of this order is beyond the time scale of political parties and exceeds the sectional interests of farming organisations. But it is vital if agriculture is to contribute more substantially to the solution of our economic problems. Some of the major topics to be considered in such an enterprise are out-

Agriculture and nutrition

Almost overnight the scientific community at large has discovered of much more attention. Sir Joseph Hutchinson argues for a major (left). Professor Jean Tremolières points to the need to stimulate Fr

lined here, in the hope of encouraging interested people to get together, to study agricultural policy in depth, and to make known their conclusions.

● **Cropping pattern.** Cereals are the basic foodstuffs of the human race. In the British diet we consume approximately 90 kg a head a year directly, and use about 360 kg a head to feed to livestock which produce meat, milk and eggs. For this we grow 13–15 million tonnes and import 8–9 million tonnes. How would one implement a decision to (a) halve, or (b) eliminate cereal imports? This would fall under two heads; first, the replacement of imported cereals used in human food, chiefly by growing good milling instead of low quality winter wheats, and replacing some of the spring barley crop with good quality spring wheats; and, second, the reduction of cereal-based livestock production to match the reduction in the availability of feed grain. Of this we have the experience of the Second World War, when pig and poultry stocks were drastically cut back, and beef production was limited to the available grass feeding acreage.

Other crops would not be so directly affected. Potatoes would probably remain about as at present. There might be considerable debate about sugar beet, but considering the need of the Third World for access to industrial markets, and the disfavour with which sugar is regarded by nutritionists, it seems likely that the crop should be stabilised at a level appropriate to use as a rotation crop in arable farming.

In a diet with a lower meat content, vegetables might have a larger place, and vegetable crops might be increased. Oilseeds are almost entirely imported, and oilseed crops should be reviewed. Ley breaks in arable rotations would assume greater importance, and are considered in relation to the maintenance of fertility.

● **Fertility levels.** The present high fertility of British farm land has been achieved by the liberal, and at times almost profligate, use of fertilisers. Now that these have become expensive and scarce, there is no doubt that considerable savings can be made by more discriminating fertiliser practice. Greater savings are to be gained by better distribution of animal manure,

and by the use of leguminous crops to augment the nitrogen supply. These two are interrelated, since the tendency to separate crop production from animal industry has resulted in an inconvenient concentration of animal manure on livestock farms and an abandonment of grass/legume leys on arable farms.

The redistribution of crops and stock in order to maximise the conservation of fertility would be the most important and the most difficult consequence of this kind of reappraisal of British agriculture. The rundown of intensive livestock enterprises would involve writing off capital in intensive housing, and the reintroduction of grazing livestock on intensive arable farms would require investment in fencing, water supply and some winter housing. How much? At what rate? And where?

● **Human nutrition.** Human nutrition lags behind animal nutrition on account of the difficulties of experimentation with human subjects. The diversity of opinion among eminent nutritionists as to the causes of the 'diseases of affluence' is sufficient indication of the uncertainties of the subject. Nevertheless, the existence of diseases associated with affluence is not in dispute, and this, together with economic need and the limitations of world food supply, is a powerful reason for devising diets that are less demanding on resources, and perhaps less menacing to health.

In conclusion, these are three major areas in which we have substantial room to manoeuvre. If we are to exploit the freedom of choice open to us with a view to realising the fullest potential of our agricultural resources, some of us must get down to the hard work of studying these topics, and charting a course of action in detail. Some groups are already concerned in this area, notably the Joint Consultative Organisation recently set up by the Ministry of Agriculture, Fisheries and Food to give guidance on research priorities, but we need a wider assessment of the place of British agriculture in British economic policy. Are we interested to provide a factual basis on which informed opinion can be built, or are we content to stumble from Price Review to Common Agricultural Policy negotiation without real understanding of where we are going? □

unfashionable subjects are worthy of British agricultural policy and nutritional research (right).

THROUGHOUT the crises of our time—the financial crisis, the troubles of society, the energy crisis—our idol, science, has remained unquestioned. Now the time has come to ask ourselves what science can contribute.

This is what has just been done in the field of human nutrition by a British committee convened jointly by the Medical and Agricultural Research Councils (MRC and ARC) under the chairmanship of Professor Neuberger. Its report is the collective testimony of a number of scientists with long and converging experience. Until now nutrition has appeared to be limited to certain specialised functions, to the study of deficiency diseases and to dogmatic ideas on diet. In fact, nutrition is not just a particular function of living beings; together with immunology and genetics, it is one of three universal concepts which relate human beings to their environment and to the society in which they live. There was a need to restore nutrition to its true dimension as a synthesis of sciences, a culture which stimulates and fertilises, and to get away from an approach which had become too purely analytical.

Why should it be surprising that it is the bread man eats which serves as the starting point for taking a new look at our attitude to science?

The disorganised state of our knowledge of nutritional science could not last. Nutritional requirements as laid down by the expert committees are a mass of contradictions, arising from a scarcity of facts which in any case have little meaning. For example, energy requirements represent the actual consumption of the reference man; yet this Apollo is a man of our industrial society whom we know to be too fat and who eats too much. On the other hand, protein requirement has a physiological basis, the minimum values having been derived from measurements made in the laboratory during brief experimental balance periods. No society actually lives at this level, however, since if energy requirements are met, even the simplest diet would provide surplus protein according to these standards. The values recommended for vitamins are saturation values which have no clear physiological meaning. Hypovitaminosis is rarely seen now, but nevertheless people are trying to use these recommended values

as a basis for labelling and advertising of dietetic products in the USA and in France. Supplementation with amino acids or iron has mostly been based on economic considerations.

In the field of diseases associated with nutrition, the inability of classical physiology to explain obesity is obvious. Measurements made over 10 minutes are extrapolated to 24 hours. The physiologist applies the law of surface area which is only valid to $\pm 30\%$; yet a systematic error of 10% would represent a weight change of 13 kg a year. The cause of degenerative disease of the heart and blood vessels has been attributed variously to the percentage of calories from fat, the ratio of EFA to NEFA, to sugar and to cholesterol. Yet this is a global state, a multifactorial syndrome, in which the causal factors are interdependent.

It is the ideas expressed in the Neuberger Report, the lines of thought, the strategy, which are important, not the details. The sergeant-major will always find something wrong with the buttons: there are no references, and some important points about dietary behaviour have been neglected, but the positive side of the report is such a relief and offers such hope that one can overlook the omissions. Doctors, biochemists, physiologists, agronomists, toxicologists, dietitians, the food industry and sociologists have all co-operated and succeeded in producing a unification of knowledge which is a truly significant synthesis.

This group of academics has emphasised the 'social' function of research in relation to the health of the individual and of the community and has tried to apply this research to a number of social and medical problems. To those of us who have lived for the last 10 years with the research policy of the Institut Nationale de la Santé et de la Recherche Médicale and of the Conseil National de Recherches Scientifiques, this report under the signature of Professor Neuberger is a bomb which will breach the ramparts.

The evidence given here shows nutrition as a special area of interaction between man and his environment. The problems of toxicity and pollution are looked at from the biochemical and physiopathological point of view and the metabolism of foreign substances has been dealt with in the same way as that of nutrients.

It is, however, at the molecular and cellular level of organisation that the most practical problems of medicine, of toxicology, of *l'alimentation* have to be solved. Again it must be realised that it is the synthesis, the common language, the ideas, which will allow specialists in each sector of this huge spectrum to understand one another clearly in order to produce the illu-

mination of which man and his society are in need.

Some of the principal nutritional problems have been mentioned as illustrations. It is not possible to summarise what is already a summary of 180 pages. This kind of committee does not usually adopt an attitude so widely based on biology, so critical and so positive. There are certainly many points for discussion, but there are so many positive suggestions that all nutritionists will be obliged to meditate on them. One is more stimulated by this report than by a classical scientific publication. It seems to me very possible and desirable that different national committees should take this document, study it in depth, fill the gaps and propose different approaches to certain points.

It is appropriate here to mention that the French effort, small though it is, operating through the Fondation Française de Nutrition and the Committee on Human Nutrition of the Centre National de Coordination des Etudes et Recherches sur la Nutrition et l'Alimentation, has embarked without previous consultation on a programme which, in proportion to its resources, is completely in the same spirit.

The topics selected are identical: nutrient requirements, an appraisal of the states of overnutrition (cardiovascular disease, obesity) and of undernutrition, a study of nutritional factors in infancy in relation to prevention of disease, toxicological aspects of food safety, and the dietary behaviour of individuals and communities. The spirits are ready and willing to work together; the means remain poor. The report from the UK is going to catalyse the timid French efforts.

To go even further, it would be desirable for Britain and France jointly to hold meetings and symposia on nutrition (once or twice a year to start with) at which points of view and communications of original work could be presented. The Nutrition Societies, the public bodies such as the MRC and ARC in the UK and the CNCERNA in France and the Nutrition Foundations could provide the impetus for such an arrangement. The first step would be the formation of a working group, aware of the present deficiencies of the subject and of the necessity to keep in touch all the time with the practical implications of food production and the maintenance of health and with fundamental research and the proper role of administrative and financial structures. The ultimate object would be to inspire research, and thus to develop a knowledge to use as a means in the service of the well being of the individual and the community. □



Marshall: energies a legend. (Financial Times photo).

build its reputation with a new and far more commercial approach, in face of great scepticism from the scientific community as well as from private industry. They devised the kind of formula that can work only between two people in closest rapport, to avoid mutual interference within each other's parish in advising the Secretary of State for Energy.

Walter Charles Marshall, 43, joined Harwell from Birmingham University in 1954. Just six years later he became Head of Theoretical Physics, and in 1966 Deputy Director. Two years later he was appointed Director of Harwell under Dr J. B. Adams, then Board member for research of the UKAEA. When Adams resigned the following year to take charge of CERN's plans for the 300 GeV accelerator, Marshall was appointed Director of the Research Group of the UKAEA. In 1972 he became its Board member for research, with full control of its research budget of £25 million.

By this time the prodigious energies of the burly young Welshman were already a legend—and not only at Harwell. In 1969 Dr Alvin Weinberg, then Director of Oak Ridge National Laboratory, reflecting upon a period Marshall had spent with him in his early thirties, remarked to me that had he remained much longer he would have taken over Oak Ridge.

But Harwell, by the mid-1960s, was presenting a challenge of a different kind. It was a research centre searching for a new mission. The basic physics and chemistry required to make nuclear weapons and lay the foundations for a nuclear power programme had been taken up by other laboratories, more firmly orientated towards engineering requirements. Harwell, some said, was becoming increasingly irrelevant. (*Nature* itself, in an editorial in October, 1967, argued that it should be closed down.)

One course of action open to the UKAEA was to cut the Research Group—then some 6,000 strong—back to a size commensurate with the mission remaining, mostly work for the fast reactor. This implied a reduction to about one-third of its size. The Government itself was well aware at the time of one drawback to this course of action, that those industries most urgently in need of new technical talent, such as the mechanical engineering industries, were the least likely to be enthusiastic about hiring redundant Harwell staff. Marshall and his colleagues, however, raised another important reservation—that a Harwell shrunk to one-third of its size simply would not be up to the very demanding business of nuclear energy research and development. So many questions remained unanswered about the fast

Marshall the energy

A profile of Eric Varley's Chief Scientist by David Fishlock

THE late Sir John Cockcroft, founder-director of the Atomic Energy Research Establishment, Harwell, in the early 1950s, was offered the post of Chief Scientific Adviser to the Cabinet. He accepted but retained his job as director of one of Britain's biggest research centres. Few today even recall that Cockcroft ever held the post in Whitehall, so slight was his influence in the broader affairs of government. Dr Walter Marshall, present Director of Harwell, was expressly warned of this unhappy precedent when, last summer, Mr Eric Varley, Secretary of State for Energy, offered him the post of Chief Scientist to his science-starved department. Marshall, however, would nonetheless accept only on condition that he retained his job as Director of Harwell, Britain's—if not Europe's—most powerful energy research and development centre.

For Marshall, still a theoretical physicist at heart, whose own research earned him election into the Royal Society while still in his thirties, the most worthwhile job in the world is to be chief executive of this big science machine. Knowing that this is so is one important reason why morale

is so high among the 5,000 staff of the Research Group of the UK Atomic Energy Authority (UKAEA), for which Marshall is the Board member responsible. But the Whitehall post, in which he would take a seat alongside the four existing deputy secretaries under Sir Jack Rampton in the Department of Energy, could expand vastly his influence over energy research and development in Britain. Coordination in this area, at a time when the emphasis was shifting from competition to conservation, would plainly be an important part of the role of government, as it became increasingly deeply embroiled in the activities of private as well as state-owned energy industries.

Even so, the twin jobs of advising both the Energy Secretary and his own boss Sir John Hill, Chairman of the AEA, could begin to work only if there existed a special relationship between Hill, as the Energy Secretary's adviser on the largest single sector of energy research and development, and Marshall, as his personal research adviser. But Marshall holds Hill in high esteem, not least for the support given Harwell during the difficult years when the laboratory was attempting to re-

reactor that nobody could possibly predict, when trouble struck, which of its vast array of techniques and talent would be needed.

Marshall and his colleagues were vehemently opposed to either closure or a major reduction in strength. As he put it in a paper published by the National Academy of Sciences last year: "When a group of talented scientists and engineers have been brought together into a national laboratory and they have partially fulfilled their original mission, it seems to me unimaginative and wrong simply to disperse the ability . . . national laboratories are a national asset, but they should be encouraged to change their orientation from time to time".

In other words, Harwell was seeking a new national mission to augment a diminishing role in nuclear fission. The most obvious subject was nuclear fusion, but this had already been hived off to a new centre, the Culham Laboratory; while Marshall himself, once deeply into the physics of fusion, had turned to solid state physics because of frustration with the chaotic state of plasma physics in the mid-1960s.

The mission conceived by Adams, Marshall and their colleagues was born of an awareness that "British scientists regularly produced good scientific ideas which led fairly rapidly to an improvement in the balance of payments of other countries but not of our own" (*The New Scientists*, OUP, 1971). At Harwell they had the skills, the equipment, and the proven ability to organise these facilities into effective problem-solving teams. The mission they sought was the freedom to offer these facilities to industrial companies, on an exclusive basis, in an endeavour to help them to innovate.

The Labour Government, well aware that Harwell was only the first of a number of national laboratories whose original missions were drawing to a close, looked kindly on Harwell's idea. It even endorsed the proposal that Harwell should flout all traditions of British government-funded science and instead of making its research freely available to all-comers (as taxpayers) should work exclusively with chosen industrial partners. With characteristic irreverence the idea was dubbed "the principle of maximum unfairness".

The difficulty, as Marshall records in *The New Scientists* was the "large number of arguments against the general idea, mostly drawn to our attention very forcibly by other people." Harwell's own attitude at the time was somewhat ambivalent, with many of the physicists and chemists opposed to the scheme, though the metallurgists, engineers, corrosion experts and so on were strongly in favour.

Other opponents included industry itself, whose response was entirely unambiguous. "Industry simply did not want us, and most people sought strongly to discourage us."

Marshall must take a major share of the credit for the success of the 'Harwell experiment' in which the portion of its income outside the Atomic Energy Vote has grown steadily to some £8 million last year, about 40% of the laboratory's budget.

Marshall's industry-orientated research effort has, however, had its full share of disappointments and failures. A prototype isotope-powered heart pacemaker failed in a patient. A promising new method of desalinating sea water was turned down by the government as uneconomic when funds were sought for a demonstration plant. Perhaps most disappointing of all was the decline and fall of carbon fibre.

Last summer he shouldered the task of bringing the deductive logic of science to bear on the affairs of the new energy department. In terms of influence the post had some obvious attractions, for the department is nominally responsible for an outlay on energy research and development which this year will total £120 million. The post also provided a seat at the table of departmental scientific advisers counselling the Cabinet on scientific affairs.

The obverse of the coin is that the research resources of the energy department are unquestionably undernourished. Most of the £120 million is administered by the various energy industries and agencies, as the accompanying table shows. As Marshall told the Select Committee on Science and Technology recently, his freedom for action in terms of research funds is restricted at present to a mere £200,000—the cash allocated last spring to set up the Energy Technology Support Unit at Harwell, which is appraising alternative energy options open to Britain.

But as Chairman of ACORD, the Advisory Committee on Research and Development for the energy industries—traditionally an independent appointment but one Marshall insisted on taking himself—he can encourage a spirit of cooperation where once there was fierce competition, and of concern to conserve rather than to promote further use of particular fuels. For the moment the emphasis is on technical services and what the application of research data already existing—perhaps garnered a decade ago—can do quickly to reduce energy consumption in industry.

Outside ACORD, to which each of the state-owned energy industries will be submitting its annual report on research for appraisal this spring, the

Department of Energy is spending about £21 million in the current year on research contracts placed with Government and industrial laboratories. Half of this sum is committed, however, to the Anglo-German-Dutch gas centrifuge project for uranium enrichment. The only laboratory that came under the direct control of the department's Chief Scientist, namely the Safety in Mines Research Establishment, was transferred to the new Health and Safety Commission earlier this year.

Several problems therefore loom large this spring for Dr Marshall. First, he has a major part to play in preparing a paper for the Cabinet putting forward the case for a new phase in the development of the fast breeder reactor, far and away the most rewarding prospect in sight for research and development in energy conservation. The new phase, however, will involve a sum of the magnitude of Britain's contribution to Concorde, for it must include a large scale demonstration power station using the sodium-cooled fast reactor.

Second, he has to coordinate Britain's role in the energy research programme of the International Energy Agency which—largely, it is said, because of his own influence—blossomed abruptly in January with a series of proposals for multi-national ventures, with Britain taking the lead in coal technology.

A third and possibly less tractable problem for Marshall is to create a research arm under his own control to the department's Offshore Supplies Office in Glasgow. Only when thus equipped will the department feel competent to monitor what must surely be a rapidly burgeoning budget for offshore and seabed research and development contracts placed with other British laboratories.

Fourth, he must strengthen his own scientific secretariat in the department, numbering merely 30 at present, and strongly orientated towards coal—a legacy of its origins in the erstwhile Ministry of Fuel and Power.

A scientist on the Cabinet Office committee of departmental chief scientific advisers has remarked that Walter Marshall tends to raise eyebrows among his older and—in terms of Whitehall politics—much more experienced colleagues by starting his contributions: "I don't know what you fellows think but at Harwell . . ." Marshall is proud of the way he has rekindled the vitality of this research centre, transforming it into a brisk, businesslike organisation, second-to-none in the management of research projects. Nobody would be very surprised if Walter Marshall now turned his top Harwell management loose on the problem of reshaping the nation's energy research and development effort.

international news

In a move that will do little to enhance public confidence in environmental regulations and even less to restore public faith in technology, the Environmental Protection Agency (EPA) last week reluctantly decided to give automobile manufacturers at least another year in which to make further reductions in car exhaust emissions. The agency really had little choice in the matter.

The basis of the decision—which EPA Administrator Russell Train said last week is “the most unhappy decision that the agency has had to take since I have been here”—is relatively simple. The catalytic converters which all American car makers have adopted to clean up exhaust pollutants may themselves pose a hazard to health, so the EPA has decided to see how serious the hazard may be before forcing even more widespread use of catalysts. In short, although the catalysts seem to do a good job in oxidising hydrocarbons and carbon monoxide to less harmful compounds, they are also quite effective at oxidising sulphur dioxide to sulphuric acid, with the result that cars fitted with catalysts may be giving out harmful amounts of acid.

The decision to put off enforcement of stricter regulations was politically painful because it now provides car

Car catalysts pose health problems

from Colin Norman, Washington

manufacturers and other industries with excellent propaganda material to use against any legislation which forces rapid introduction of clean-up technology. When Congress belatedly forced the car companies to clean up exhaust emissions by putting a pistol to their heads in the shape of the Clean Air Act, the only technology available was the exhaust catalyst. The car companies screamed that catalysts would be expensive, would increase gasoline consumption, and would simply be bad technology. They begged for more time to develop other technologies, but Congress and the EPA decided that, on the basis of past performance, the car companies wouldn't use the time to develop other means of controlling exhaust emissions, and so forced them to use catalysts.

As far as hydrocarbons, carbon monoxide and oxides of nitrogen are concerned, the catalysts have proved to be

extremely effective. What's more, in spite of Detroit's warning to the contrary, their introduction on many 1975-model cars has even led to increased performance and efficiency. Then came the sulphuric acid problem.

It first came to light in the mid-1960s, but was disregarded as being insignificant. Then, in mid-1973 the problem was brought up again and given widespread publicity, and the EPA started a crash programme to determine just how serious a hazard to health it might be. The EPA found that cars equipped with catalysts produce, on average, 35 times more sulphuric acid per mile than cars without catalysts. Moreover, if an air pump is used to inject more air into the catalyst—a device which would be required to meet stricter pollution control standards—then production of sulphuric acid would be about 70 times that of a car without a catalyst.

Although the amount of acid produced by automobiles would be only a tiny fraction of that produced by factories, the problem is that it would be concentrated along freeways and in cities.

It wouldn't be so bad if there were a potential solution just around the corner, but there isn't. None of the three possible solutions—removing sulphur from gasoline, putting a sulphur trap in the exhaust and improving catalyst technology—provides a basis for regulation, Train said last week. It therefore seems at this stage that the only real chance of removing harmful pollutants from car exhausts without causing another health problem is for the car companies to go for a completely different engine design, such as the stratified charge engine. But since Detroit produces 10 million cars a year, it would be many years before the entire production line could be converted to a new technology.

At least total amounts of automobile pollutants spewed into the atmosphere will continue to decline, even with the regulations frozen at this year's level, because older, dirtier cars will be replaced with cars equipped with 1975-level control devices. But, since the fight over automobile pollution has been widely publicised, and because Congress and the EPA staked their hopes on the catalytic converter, the utter shambles that now reigns will be greeted with glee not only in Detroit but in a good many other corporate board rooms as well. □

THE US Fish and Wildlife Service believes it has found a cheap and effective method for removing the potent teratogen dioxin from the herbicide 2,4,5-T. If so, it would remove some, though not all, of the opposition to use of the herbicide in the United States, and it would also provide a solution to the problem of what to do with the 2.3 million gallons of dioxin-contaminated 2,4,5-T left over from the Vietnam war.

The process is extremely simple. According to an announcement from the US Department of Interior, all that is required is to filter the solution through coconut charcoal and to rinse the charcoal with acetone. Tests carried out at the Fish and Wildlife Service laboratory at Columbia, Missouri, have shown that more than 99% of the dioxin in 2,4,5-T is removed by the method, and the government has applied for a patent for the process.

Although removal of dioxin from 2,4,5-T would make the herbicide much safer, there is considerable doubt about whether or not 2,4,5-T itself is terato-

genic. The herbicide is therefore still unlikely to get a completely clean bill of health from its critics.

● After that embarrassing incident last year when President Ford had to withdraw the nomination of Andrew Gibson as head of the Federal Energy Agency when it became known that Gibson was drawing a large salary from the oil industry, Presidential appointees have been subjected to a thorough investigation before their names are sent to the Senate for confirmation. Last week, however, Ford announced that four people have passed the test and are being nominated for top science posts. They are Robert W. Fri, to be Deputy Director of the Energy Research and Development Administration (ERDA); James L. Liverman, to be Assistant Administrator of the ERDA for the environment; John Teem to be an Assistant Administrator of the ERDA for advanced energy systems; and Richard C. Atkinson to be Deputy Director of the National Science Foundation.

THE Medical Research Council is to set up a new unit for research in clinical oncology and radiotherapeutics at Cambridge; at the same time, the Cancer Research Campaign has provided an endowment of £275,000 to establish a new university chair of clinical oncology. Professor Norman Montague Bleehen has been appointed Honorary Director of the MRC Unit and Professor of Clinical Oncology. He will take up both posts at the beginning of the academic year in October. The unit officially starts operation in October and will be provisionally housed at the new Addenbrooke's Hospital. It is hoped that purpose-built accommodation can be provided within five years. Professor Bleehen is at present Professor of Radiotherapy at the Middlesex Hospital. Under his direction, the new unit's general aim will be to improve the results of current methods for the treatment of cancer and to develop improved methods of measuring the clinical and biological response of tumours to treatment. The programme will include investigations on lung cancer, rectal cancer and osteosarcoma, with particular reference to occult metastatic disease. Staff at the new unit will collaborate closely with other workers there with oncological interests. In particular, it will provide a clinical link for the laboratory research now being carried out in Cambridge by a Tumour Biology Group led by Dr Sydney Brenner at the Laboratory of Molecular Biology.

The new chair in clinical oncology is the fourth to be funded by the Cancer Research Campaign. Chairs have already been set up at the Institute for Cancer Research in London and at the universities of Manchester and Glasgow. A fifth is to be established shortly at the University of Southampton, and when complete the scheme will have cost about £3 million. The Cancer Research Campaign is the largest supporter of research into cancer, including leukaemia, in the UK and in 1974 awarded grants totalling more than £4.5 million.

● In this year's list of prizes and medals awarded by the Institute of Physics, aficionados of the entertaining experimental demonstrations at the Royal Institution lectures will be glad to see the award of the Bragg Medal and Prize to Mr W. A. Coates "for his contribution to education through the design and execution of demonstrations illustrating the many lectures held at the Royal Institution". Mr Coates first joined the Royal Institution in 1948 as a technical research assistant on X-ray diffraction experiments and became Senior Experimental Officer in charge

of preparation and production of teaching demonstrations under the directorship of Sir Lawrence Bragg.

After more than 10 years organising the demonstrations, which Mr Coates admits he embarked on with some reluctance when Sir Lawrence asked him to help with the lectures for schools started in the late 1950s, Mr Coates still thoroughly enjoys a job which can have its unexpected hazards. Recently, after setting up a demonstration for measuring the potential of an electric eel, an ugly customer which can give a shock up to 400 volts, he returned from a meal to find the tank empty and the eel gone. Luckily it had landed on a rubber mat nearby and so was returned to its tank with no further mishap; the demonstration was a complete success.

Round Britain

● The publication this week of the latest annual report of the Science Arm of the Agricultural Development and Advisory Service (ADAS) of the Ministry of Agriculture (HMSO, £5.50) should prove a good reference book for agricultural research scientists looking for sources of useful but often unpublished data, and of problems which would repay further research.

In their role as scientific troubleshooters to the agricultural industry ADAS has amassed a unique collection of field and experimental data (which of its nature is usually not publishable in the conventional scientific literature) on a wide variety of agricultural problems ranging from heavy metal contamination of sewage sludges used as fertilisers and water pollution by agricultural wastes, to the optimum feed formulations for cattle. As well as highlighting problems in need of further research, such data will prove invaluable in drafting pollution legislation, for example.

Much of this work had previously been hidden in internal reports but with the formation of ADAS in 1971 from several of the ministry's advisory services, it was felt that the work could be usefully brought to the attention of a wider audience. Formal links between ADAS and the Agricultural Research Council have also been strengthened recently with the formation of the Joint Consultative Organisation for Agricultural Research Policy, on which ADAS is represented.

Britain's entry into the EEC has also involved ADAS in the collection and evaluation of technical information and advice for the negotiating teams in Brussels trying to harmonise the many regulations concerning agricultural

practice and produce throughout the community—for example those dealing with the antibiotic content of animal feeding stuffs and the tests used to determine the quality of milk and milk products. The service has also been involved in the analytical tests to determine standards of produce submitted to the Intervention Board for Agricultural Produce.

● Professor Jack Meadows, of the University of Leicester, has been given a £16,000 grant by the British Library to study how scientific ideas get to the mass media and how they are used by television, radio, newspapers and magazines. Part of the grant will be used for research into the way in which journals monitor research papers and why some work is rejected and some accepted in a situation in which the journal is receiving more 'good' papers than it can publish.

This work stems from a study in the United States by Merton and Zuckerman, who monitored the rejection rate of the leading specialised journals over a range of subjects and found a definite correlation with subject. Journals of the physical sciences had lower rejection rates compared with biological journals, and the rate was even higher for psychology and learned journals of the humanities. The American workers also found that the rejection rate was positively correlated with the degree of disagreement among the referees.

Professor Meadows and his team hope to extend this type of investigation into the British system, as the influences at work may well be different from those in the United States. He hopes to find out in detail what happens in the refereeing process, and whether some of the myths surrounding refereeing are in fact justified. Needless to say this type of investigation must be organised so that the confidentiality of the refereeing system is maintained. In tune with the times, the team will also investigate how the economic situation, as reflected in decreases in the number of journal pages, has affected the refereeing process by perhaps making it more stringent.

● Much head-scratching and pen-chewing in Cambridge these days. Professor J. W. Linnett, Vice-Chancellor and Professor of Physical Chemistry, is worried that university research is being poor-mouthed by certain 'ladies and gentlemen'. So he has circularised departments to enquire of any "useful research . . . of clear value in the relatively short term". If the right sort of stuff comes up he then intends to alert ministers, MPs, newspapers and industrialists to the merits of the place.

LAST year Israeli farmers produced 78% of the foodstuffs consumed in Israel, as well as exporting enough agricultural products to pay for most food imports. Now, with the active assistance of local researchers, they are attempting to reduce further Israel's dependence on imported food products, particularly commodities like wheat and sugar which have gone up in price by hundreds of per cent in recent years.

Research has already been a key factor in raising the average yield of wheat from some 900 pounds an acre in 1950-55 to more than 1,600 pounds an acre today, with yields of 6,000 pounds an acre common in more fertile parts of the country. Current studies could well increase output even more.

Some of the most interesting work is being carried out by Moshe Feldman and Dan Atsmon, of the Weizmann Institute, who are using sophisticated cytogenetic techniques in an attempt to transfer valuable characteristics from native Israeli wild wheat to standard wheat varieties being grown here and elsewhere.

Since this area, long known as the Fertile Crescent, is the cradle of wheat farming, it naturally abounds in wild varieties. And although these are not satisfactory for modern farming themselves, they nevertheless manifest some very interesting characteristics worth "stealing".

Drs Feldman and Atsmon are paying special attention to a species from the Negev Desert (*Triticum longissimum*), which is at home in an area with as little as 25 cm of rainfall annually, tolerates brackish water, and grows and matures quickly—so much so that, were cultivated wheat to ripen as fast, it would be possible to plant and harvest two (irrigated) crops a year.

The Weizmann scientists have isolated seven individual chromosomes of the Negev wild wheat, including the one responsible for its early maturation. Now they are attempting to incorporate selectively these valuable chromosomes into standard wheat varieties, which should make it possible to increase yields in well watered areas, and also permit wheat to be grown in other areas where sparse rainfall has so far prevented its cultivation.

More land will likewise be given over to the growing of sugar beet, while research continues in an attempt to develop other economically viable sources of sugar and sugar substitutes. Ministry of Agriculture studies have indicated, for example, that at present price levels it is worth making sugar from locally grown sorghum.

Meanwhile scientists at Ben-Gurion University of the Negev, led by Pro-

fessor Jaime Wisniak, are concentrating on sugar substitutes, including xylitol. So far produced on a commercial basis only in Japan, xylitol is derived from vegetable residues such as cotton seed hulls, corn and other seeds containing cellulose. It has the same crystalline form as sugar and is actually 10% sweeter. Since it does not require insulin for metabolism of body material, it can safely be used by

cause damage.

● Religion, or more properly religious folklore, provided the name for Israel's largest locally built computer, the Weizmann Institute's Golem. The original Golem was a legendary automaton created by a famous Prague rabbi in the Middle Ages and, like Mary Shelley's Frankenstein, it eventually turned on its creator.

The legend came to mind last week when 30,000 Israeli primary school teachers declared what may have been the world's first strike against a computer. Their one-day walkout was called to protest about difficulties with the Ministry of Education's computer which have caused long delays in the payment of salaries for the past 16 months.

Computing teachers' salaries does present special problems, as 18,000 changes must be taken into consideration every month. But these problems could easily have been solved if it had not been for the human factor.

Trouble began in the Ministry of Education's Data Processing Department some four years ago when it became clear that the rented IBM 360/40 then being used to calculate salaries was no longer able to handle the work load. Department employees suggested that it be replaced by a higher capacity IBM machine, which, coming from the same company, would ease conversion problems. The ministry decided instead to purchase a Burroughs computer because it was \$250,000 cheaper and because, according to benchmark tests carried out in the USA, it was able to perform tasks required by the ministry about 15% faster.

Preparations for conversion to a Burroughs computer were carried out in the department while regular work was done on the old IBM. Difficulties arose, but only became really serious when call-ups because of the Yom Kippur War took away 80% of the data processors. By the time they returned there was near chaos in the salary section, prompting employees to argue that only the introduction of a new IBM could save the situation. When the ministry remained unmoved, 46 of the 47 people in the Data Processing Department handed in their resignations and, according to Education Ministry Director General Elad Peled, left without passing on vital information about tape codes for salary calculations to their replacements (provided by the local representatives of the Burroughs Company).

Peled charged his former subordinates with sabotage; they, in turn, charged him with libel. But all this is of little interest to teachers, who merely want their salaries on time.

Letter from Israel

from Nechemia Meyers



Western Wall: to weed or not to weed?

diabetics. Wisniak proposes that Israel produce xylitol on a large enough scale to meet domestic requirements and to allow for exports.

● Israeli scientists studying plant life are involved not only in solving the country's food problems but also some of its religious problems. Namely, they are being asked by religious authorities whether or not weeds should be pulled out of the sacred Western Wall in Jerusalem's Old City.

Diametrically opposed rulings have been issued by Israel's constantly feuding Chief Rabbis, Shlomo Goren and Ovadia Yosef. Ashkenazic Chief Rabbi Goren declared that the plants could not be pulled out since they are symbolic of the destruction of the Second Temple (in 70 AD) and the longing of Israel for redemption. Sephardic Chief Rabbi Yosef ruled that they could be pulled out, particularly as they might crack the Herodian-period stones.

The man who asked the Chief Rabbis for their opinions in the first place, Rabbi Dov Perla of the Religious Affairs Ministry, has now turned to a botanist to determine—for the first time in the Wall's 1,900-year history—whether or not the weeds are likely to

Flood studies from NERC

from Angela Croome

THE engineer designing a dam or a reservoir, or the city planner contemplating urban development on river embankment or flood-plain is in the hands of the hydrologist and to some extent the meteorologist. Britain's Institution of Civil Engineers (ICE) grasped this fact in the 1930s after a major review following the last failure of a dam in Britain—happily now 50 years ago (which is something of a record). The institution has pressed ever since for a comprehensive applicable prognostic approach enabling engineers to design with confidence a structure which would last. Britain's civil engineers obtained this document last week in the form of the Natural Environment Research Council's *Flood Studies Report*.

A draft code of practice closely associated with this report is coming out for discussion within a few weeks, and from some time in 1976, when adopted in final form, any civil engineer in Britain who does not follow it will be guilty of professional negligence. In May the ICE is calling a meeting in London to examine in detail the report and the code of practice, their validity, and their relevance outside Britain.

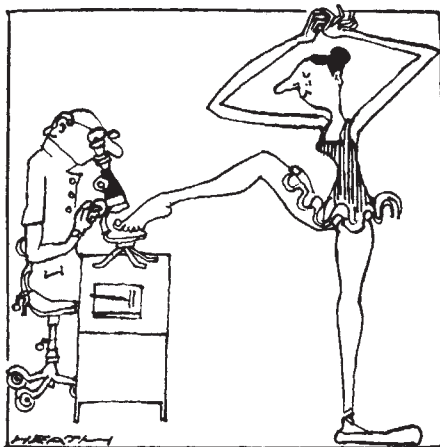
The influence of the report is likely to be great and much more widespread than its original terms of reference might suggest. It is the most comprehensive attempt that has been made anywhere to tackle the problem of flood estimation and, in the opinion of the International Commission on Large Dams, it is "head and shoulders" above all others. The ICE is already prepared to say that as a result of its findings many flood-protection schemes will have to be revised.

The study has involved nearly five years of work by a multidisciplinary team based on and led by a staff of 15 recruited by the NERC Institute of Hydrology, and assisted by a six-man group of Meteorological Office scientists. The statistical analysis of correlated rainfall and runoff records on the widest possible scale forms the core of the study. This has involved collecting an archive of 9,300 'station-years' of records (from the 1,200 stream-gauges in the UK) plus a further 1,700 'station-years' from 110 Irish gauge-stations. Records from 6,000 rainfall stations were also included.

The result in practical terms is that for any area of 500 km² in Britain, a reliable estimate of the mean annual flood level and its frequency can be obtained. (In other terms, the report amounts to the most comprehensive

available textbook on hydrological statistics—for all the data is tabulated there as well as the mathematical relationships derived from them.)

Basically, there are two methods now available. Where there are gauge-station records for a given river basin, a statistical method is presented enabling the practitioner to get specific answers to his questions—how high is the maximum flood level he must build for over, say, 10,100 or 1,000 years, and how often is it reached. This can be derived either from records of the annual maximum for the area or from data on the peaks above a threshold level. Where only rainfall records exist—that is, in an ungauged river basin—the run-off characteristics of a site can be estimated, given information on four characteristics of the catchment, namely, slope, soil, land-use and area. As rainfall records are usually more extensive and go back further, the second method may be more easily applicable outside Britain but it can only be reliably applied in other cool or temperate regions. □



THE results of a 15-year survey, carried out on more than 100 pupils of the Kiev School of Choreography, has revealed significant modifications in the toe structure of ballet students; these are put down to the increased loads imposed on the toes when dancing on the point or demi-pointe. The study, carried out by biologist Vladislav Pelipenko and based on regular X-rays of the toes during the course of 15 years of training and dancing, showed that within a short time the bones undergo significant thickening of the outer layers and restructuring of the inner layers. On the basis of these measurements, Pelipenko has compiled tables which, he claims, will provide a basis for the selection of new pupils and a means of forecasting their future performance. The results of these experiments, he suggests, can also be applied to draw up training schedules for gymnasts and athletes, who also experience unusually large loads on the toes. □

Science writers and government policy

from David Spurgeon, Ottawa

PROFESSIONAL science writers and scientists co-exist for the most part under conditions of uneasy truce. Thus it is refreshing when one side finds good things to say about the other. Just such an occasion was the fifth annual science writing seminar of the Canadian Science Writers' Association, held on February 24 and 25 at the University of Toronto. The welcome by Dr George Connell, Vice-President Research and Planning at the university, was positively fulsome.

The reason, basically, was that Dr Connell felt that "active and well informed men and women of the press, radio and television" were to a large extent responsible for influencing the federal government to reverse the recent downward trend in research funding.

"For the first time in six years", said Dr Connell, "the levels of support through the [research] councils will balance inflation."

Dr Connell recalled that the buying power of the average grant from the National Research Council (NRC) in the physical sciences, the biological sciences (excluding health sciences) and the engineering sciences decreased by 7% between 1970 and 1974.

"Early in 1974 a group of medical scientists and well informed laymen decided to present to the public an account of the benefits of medical research and the difficulties which have impeded its progress," Dr Connell said. "The response of the press, radio and television to this initiative was remarkable: in Toronto the daily newspapers provided coverage which was remarkable both for its quality and quantity. The activity in other cities across the country was also notable."

The outcome, the vice-president said, was that, in the main estimates tabled the week before the seminar, the government proposed to spend \$48.4 million through the Medical Research Council (a 13% increase over the 1974-75 estimates); NRC estimates for scholarships and grants increased from \$69.3 million (1974-75) to \$81.7 million (1975-76) and the Canada Council's increase was from \$23.9 million to \$27.7 million.

The usual discussion took place of the difficulties of understanding and translating scientific jargon, but it was left to a French Canadian writer to put things in a suitably Canadian perspective: he said that being a French speaker was a much bigger communication problem than all the rest, because French-speaking scientists in Canada are so hard to find. □

correspondence

Nutritional landmark

SIR,—Reports prepared by expert committees on specific areas of nutrition are frequently issued, notably by the Food and Agricultural Organisation and the World Health Organisation. It is, however, an event of significance when a comprehensive survey of food and nutrition research in a single country with a strong tradition of scientific and medical investigation is assembled by a large team representative of the available experts. It is indeed a considerable achievement to have synthesised such a readable report from the documents provided by the various working parties, to one of which I was a minor and temporary contributor. The product is a landmark document, which appears at a time when the bridge between basic research and applied research in the national interest must be strengthened in the interests of survival. Food and nutrition research needs to attract original minds trained in the basic sciences. This document should provide the points of contact. It is thus entirely proper that the committee responsible for assembling the report should have included Sir Douglas Black, Chief Scientist to the Department of Health and Social Security and responsible in Britain for implementing contracts in areas of applied research.

I write this comment because a petulant and partly irrelevant review appeared in *Nature* (January 10) castigating the report because of insufficient emphasis on social nutrition, and ridiculing the priority areas for research identified in the report. On the contrary, the report correctly identifies and summarises current knowledge in numerous areas of nutritional research that are of great importance to human health and survival: the role of nutrition in vascular disease, diet and cancer, dietary fibre and the spectrum of diseases in a population, the aetiology of osteoporosis; nutritional aspects of renal disease, to name some in only one section of the report. The current problems in food and nutrition research are numerous, important and challenging. Further research into the areas identified in this comprehensive document should allow governments to plan research strategies, to test new food sources and to monitor the population more efficiently. Finally, the present disreputable status of nutrition research among basic scientists and the medical

profession will also be materially mitigated by the vigorous well-founded directions indicated in the report.

H. V. MUNRO

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Re-use of vials

SIR,—In the February 27 issue, Professor Born rightly drew attention to the need to minimise waste in laboratories and cited as a specific example the destruction of scintillation vials after being used only once. It may be of general interest, therefore, that in this department we have for a number of years successfully re-used vials without risking contamination or any of the other problems which Professor Born mentioned. The procedure we have adopted involves placing the radioactive sample and scintillation fluid in an ordinary 5 cm × 1.2 cm diameter specimen tube that is inserted into the vial for counting. This simple method has the following advantages: (1) the vials themselves are not contaminated; (2) the volume of scintillation fluid is only 2 ml, thus reducing costs; (3) the specimen tubes are very much cheaper than the scintillation vials. They may be either discarded or used for other purposes after normal cleaning.

The method works particularly well for non-aqueous samples—for example, materials on membrane filters or papers—since in these cases there is no reduction in either the counting efficiency or the amount of labelled material used in the assay.

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Science and technology

SIR,—In his paper on the changing relationship between science and technology (August 23, 1974) Langrish quotes evidence from previous citation studies which, he asserts, support his views that "British university research has little impact on British industry". We wish to challenge the view that generalisations can be made across all fields of scientific endeavour on the usefulness or otherwise of university research to industry on the basis of the evidence provided by citation analysis.

On the assumption that industrial

reviewers will cite any university research which they consider to be of relevance to industrial activity, Langrish chose seven review articles written by British industrial chemists in the 1967 volume of *Reports on the Progress of Applied Chemistry*, and found that the papers which the reviewers considered most relevant to technological developments in the seven areas were largely of non-university origin. The areas chosen were: phenolic resins, microbiological techniques in manufacture, water treatment, plastics, polyolefins, synthetic detergents and poultry and eggs. In only one area (microbiological techniques) was the influence of university research dominant, and this indicated to the author the importance of the universities in generating new techniques for industrial uptake.

We suggest, however, that a citation study such as this fails to take into account the fact that it is much more likely that there will be university research activity in the area of microbiological techniques, for example, than in water treatment, and that there may be certain major areas of scientific enquiry in which the institutional sources of citations are quite different from the sources found in the disparate subject-areas used as samples by Langrish. In particular, we suggest that the conclusions reached in citation studies of the physical sciences do not have general validity and, in order to demonstrate this, we carried out a study of the institutional sources of citations in five biologically oriented review articles in *Reports on the Progress of Applied Chemistry*. All the articles were concerned with well established areas of scientific enquiry, and showed that in terms of citations, the universities outstripped every other sector.

One should be wary of generalisations about the industrial utility of university research which rely on evidence from citation data. Langrish has stressed the implications of his citation data for policy makers in government and industry, and the fact that we have been able to obtain results which differ so widely from his raises serious doubts as to the validity of the technique of citation analysis in the determination of the relationship between science and technology. Policy decisions cannot and should not be made on the basis of such dubious evidence.

G. PRAGIER
J. RONAYNE

Brisbane, Australia

news and views

Viruses associated with human leukaemia

from Robin Weiss

THREE new reports strengthen the opinion that there is a C-type virus associated with human leukaemias. Mak and his colleagues at the Ontario Cancer Institute (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 623; 1975, and **71**, 4336; 1974) describe the appearance of virus-like particles in medium from short-term cultures of leukaemic bone marrow cells. Cultures of bone marrow from normal individuals did not release particles, but cultures from both lymphocytic and myelocytic leukaemic patients did so, when the bone marrow was taken either at blast cell crisis or in remission. Mak *et al.* have not been able to demonstrate budding particles, although the incorporation of tritiated uridine into the RNA of the particles indicates that viral RNA synthesis occurs in culture. The virus-like particles have the buoyant density, reverse transcriptase and 60–70S RNA typical of animal C-type viruses, and molecular hybridisation studies indicate that it is related to woolly monkey lymphosarcoma virus and to Rauscher virus. In contrast to their bone marrow cultures, Mak *et al.* found that cultures of peripheral white blood cells taken from leukaemic patients do not release virus-like particles. The Toronto group seem however to be unaware of an earlier report by Kotler *et al.* (*Nature new Biol.*, **244**, 197; 1973) that white blood cells from patients with lymphocytic leukaemia can be induced to release very similar particles when cultivated in medium lacking arginine.

Gallagher and Gallo at the US National Cancer Institute (*Science*, **187**, 350; 1975) describe the production of virus from cultures of three myeloblastic cell lines from the peripheral blood of a patient with acute myelogenous leukaemia. For the first time these authors show really convincing electron micrographs of C-type virus particles, including budding forms. The virus found in the medium of the myeloblast cultures possesses all the physical and chemical properties that characterise C-type viruses, and the reverse transcriptase activity is specifically inhibited by antibodies prepared against the reverse transcriptases of gibbon and woolly monkey viruses.

Gallagher and Gallo did not observe

virus production in white blood cells freshly isolated from leukaemic patients, but on prolonged cultivation of the myeloblasts from several cases of acute myelogenous leukaemia, virus began to be released from cultures derived from one 61-year-old patient. Perhaps the most notable advance is not the appearance of the virus itself, but is the ability to maintain long-term, exponentially proliferating cultures of myeloblasts. The growth of the myeloblasts depends on a conditioning factor produced by a human embryo cell strain. Gallagher and Gallo believe that the conditioning factor, which has not yet been characterised, is necessary both for myeloblast growth and virus release. They are confident that the virus arises directly from the leukaemic cells, since neither virus nor viral components were detectable in the embryo cell strain or the filtered, conditioned medium.

Scherr and Todaro at the US National Cancer Institute (*Science*, **187**, 855; 1975), using material generously provided by Gallo and Gallagher, now report that antigens related to a major structural protein (p30) of the woolly monkey and gibbon C-type viruses are present in the white blood cells of five patients with acute leukaemia.

While these new findings mark a significant advance in the search for human leukaemia viruses they come as little surprise. Over the last three years evidence has steadily mounted, largely from Spiegelman's and Gallo's laboratories, that a viral entity is associated with human leukaemic cells. A DNA polymerase with the properties of viral reverse transcriptase was regularly detected in human leukaemic cells (*Nature new Biol.*, **240**, 67 and 72; 1972). The enzyme was associated with high molecular weight RNA in a cytoplasmic particle but no complete, cell-free virus particle could be detected. Later, Todaro, Gallo and Gallagher (*Nature*, **244**, 26; 1973; *Proc. natn. Acad. Sci. U.S.A.*, **71**, 1309; 1974) showed that the enzyme activity of the cytoplasmic 'particles' was specifically inhibited by antisera raised against the reverse transcriptases of gibbon and woolly monkey C-type viruses. Hybridisation studies by Baxt, Spiegelman and

colleagues (*Proc. natn. Acad. Sci. U.S.A.*, **69**, 3737; 1972; **70**, 2629; 1973 and **71**, 2853; 1974) indicated that DNA complementary to the particulate RNA was present in human leukaemic cells but was not detectable in normal white blood cells, not even in two cases of identical twins to the leukaemic patients. Gallo's group (*Proc. natn. Acad. Sci. U.S.A.*, **70**, 3219; 1973 and **71**, 3177; 1974) have compared the homology of the 'viral' RNA of human leukaemic cells to a variety of animal C-type viruses and have found that it is most closely related to the woolly monkey lymphoma-sarcoma virus complex.

It was therefore predictable that the human viral particles isolated by Gallagher and Gallo and by Mak *et al.* would prove to be related to the woolly monkey virus; the human leukaemic p30 proteins described by Scherr and Todaro are indistinguishable in a radioimmune competition assay from the p30 of the woolly monkey virus.

Oncologists have become justifiably sceptical of new claims for the isolation of human tumour viruses, since some highly publicised announcements of human viruses in the past were found on subsequent analysis to be contaminating animal viruses. Clearly it is going to be difficult to prove that these latest isolates are not a result of contamination with laboratory stocks of woolly monkey virus. Nevertheless, there are several telling points against contamination. First, it is clear that freshly isolated human leukaemic cells that have not been maintained in culture carry the reverse transcriptase, p30 protein, and some RNA sequences related to the woolly monkey virus. Second, Mak's virus-releasing bone marrow cells were all short-term cultures maintained in a laboratory where woolly monkey virus was not propagated. Third, the virus produced by Gallagher's long-term cultures appeared in three sublines that were maintained separately from the first passage onwards. But it does seem strange that a human virus should have such a close affinity to a virus complex isolated from a New World monkey. To my mind, it is more likely that at least one component of the woolly monkey virus complex was acquired from man,

for it represents a single isolate from a pet monkey (Theilen *et al.*, *J. natn. Cancer Inst.*, **47**, 881; 1971). Perhaps the long sought human virus has been in our laboratories for the past four years.

The human leukaemia 'virus' does not seem to be endogenous because homologous proviral nucleotide sequences have not been detected in DNA extracted from normal human tissues, not even in Baxt's identical twin studies referred to already. There is however some recent evidence for an endogenous human virus related to RD-114 and baboon viruses (Strand and August, *J. Virol.*, **14**, 1584; 1974; Scherr and Todaro, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 4703; 1974). This virus does not seem to be closely related to the woolly monkey virus and its expression is not particularly associated with leukaemia or with other neoplasms, but rather with systemic lupus erythematosus. In cats, for instance, the infectious, leukaemia-causing virus has very little homology with the endogenous viral genome. Could a human leukaemia, therefore,

be acquired by infection, as in cats and chickens?

There is no epidemiological evidence that leukaemia is contagious, except for one recent report by Schimff *et al.* (*Lancet*, **i**, 124; 1975) of possible clusters in incidence of leukaemia of various kinds by social contact in a small rural community in West Virginia. If, however, leukaemia were a rare and late consequence of a very widespread and common infection it would be difficult to obtain epidemiological evidence for infection. Indeed this was the case with feline leukaemia before the virus was isolated and exploited as a serological tool. In concluding their paper, Scherr and Todaro state that "the isolation of complete C-type viruses from human leukaemic cells should greatly assist in evaluating their etiologic role in the disease". A number of laboratories are now attempting to propagate the viruses or particles isolated by Mak and by Gallagher and Gallo while further, independent isolates are eagerly awaited.

accumulate. Straight chain acids of this type have been found by Barley *et al.* in rat glial cells cultured in vitamin B₁₂-deficient medium (*J. biol. Chem.*, **247**, 4270; 1972). More recently similar abnormal fatty acids together with a branched chain 17-carbon version were found in autopsy tissue from a child who had extremely high levels of urinary methylmalonic acid probably as a result of a congenital inability to convert vitamin B₁₂ into its coenzyme forms (Kishimoto *et al.*, *J. Lipid Res.*, **14**, 69; 1973). And, most exciting, Frenkel has demonstrated 15- and 17-carbon fatty acids in peripheral nerve biopsies from cases of pernicious anaemia (*J. clin. Invest.*, **52**, 1237; 1973).

It is probable that the straight-chain fatty acids are formed when propionyl coenzyme A substitutes for acetyl coenzyme A as a substrate for fatty acid synthetase and the branched-chain fatty acids are made when methylmalonyl coenzyme A substitutes for malonyl coenzyme A. The big question that remains is whether the accumulated abnormal fatty acids are really the cause of the neural lesion associated with vitamin B₁₂ deficiency and if so, how? Even the more impressive of the above studies have their shortcomings. How closely do cultured rat glial cells really relate to human spinal cord *in vivo*, particularly considering that the rat does not develop neurological problems when depleted of vitamin B₁₂? Why do children with congenital methylmalonylaciduria seldom, if ever, exhibit the same neurological lesions as in pernicious anaemia? And are the abnormal fatty acids found in the peripheral nerves in pernicious anaemia also found in the spinal cord where the demyelination occurs? Nevertheless the similarity of the findings in each study are impressive enough for the problems to be pursued. The availability of the bat as a model for the neurological changes of human vitamin B₁₂ deficiency should considerably assist the pursuit.

For those who cannot work with bats there are always bugs. Work continues apace on the metabolism of vitamin B₁₂ in bacteria, if for no other reason than the pious hope that there are undiscovered vitamin B₁₂-dependent enzymes which are essential for the normal functioning not only of bacteria but of man as well. Thus methionine synthetase, the only known vitamin B₁₂-dependent mammalian enzyme apart from methylmalonyl-CoA mutase, was discovered in *Escherichia coli*. But it should not be forgotten that ribonucleotide reductase, found in *Lactobacillus leichmanii* as a vitamin B₁₂ requiring enzyme, was at one time the great white hope for the biochemical basis of the megaloblastic anaemia of human vitamin B₁₂ deficiency. Although the en-

Vitamin B₁₂ in bats and bugs

by Peter Newmark

THE neurological lesion which often accompanies human pernicious anaemia has no certain biochemical explanation. One of the hindrances to progress has been the lack of a suitable animal model. Though vitamin B₁₂ deficiency (the cause of pernicious anaemia) results in both megaloblastic anaemia and demyelination of the spinal cord in humans, no common experimental animal placed on a vitamin B₁₂ deficient diet has shown a consistent neurological lesion comparable to that in man. But according to the report by Green *et al.* in this issue of *Nature* (page 148) the Egyptian fruit bat may fill this gap.

The success of Green *et al.* may have had its origin in the natural diet of the bats. This is vegetarian and therefore ostensibly devoid of vitamin B₁₂. Green *et al.* suggest that wild bats obtain the vitamin by consuming both stagnant water and insects contaminating the dietary fruit. Both sources could be excluded in the laboratory diet of peeled fruit and tap water. Within 7 months the bats had difficulty in climbing and flying associated with histological changes in the spinal cord reminiscent of the demyelination seen in human vitamin B₁₂ deficiency.

If this model fulfils its promise it will have appeared at an opportune moment for exploitation because recent evidence has at last suggested a plausible biochemical link between vitamin B₁₂ deficiency and demyelination. The link

is based on the requirement of a vitamin B₁₂ coenzyme for methylmalonyl-CoA mutase and the consequent accumulation of methylmalonyl coenzyme A in vitamin B₁₂-deficient tissues. Excessive methylmalonyl coenzyme A, and its precursor propionyl coenzyme A, could interfere with the normal metabolism of the fatty acids of the myelin sheath, resulting ultimately in demyelination. The evidence that now makes this a distinct possibility is several fold.

Clearly there is some rebalancing in the fatty acid metabolising enzymes of vitamin B₁₂-deficient tissues. Frenkel *et al.* (*J. biol. Chem.*, **248**, 7540; 1973) have demonstrated increased activities of both acetyl-CoA carboxylase and fatty acid synthetase in the liver of vitamin B₁₂ deficient rats, possibly in response to the inhibition of both enzymes by methylmalonyl coenzyme A. The increase, at least of fatty acid synthetase, seems to be the net result of markedly increased synthesis of the enzyme together with a less dramatic enhancement of its degradation (Frenkel *et al.*, *Archs Biochem. Biophys.*, **162**, 607; 1974). It is not, however, altogether obvious how to translate such changes into likely effects on the neural tissue.

Much more impressive therefore have been demonstrations of accumulated abnormal fatty acids in neural tissue accompanying vitamin B₁₂ deficiency. In particular it is 15-carbon and 17-carbon saturated fatty acids that

zyme is not confined to *Lactobacillus* (Hamilton, *J. biol. Chem.*, **249**, 4428; 1974), there is now little doubt that in mammalian tissues it is replaced by a vitamin B₁₂-independent version.

Ethanolamine deaminase is indubitably not a mammalian enzyme. It is found in clostridial bacteria, is vitamin B₁₂ dependent and has been studied in great detail by Babor (for example *J. biol. Chem.*, **249**, 4537; 1974). Chang and Chang provide evidence on page 150 of this issue for the presence of the same enzyme in *Salmonella typhimurium* and perhaps also in other enteric bacteria. The enzyme, like the clostridial one, is probably induced as a response to the supply of ethanolamine as the sole source of carbon and nitrogen together with vitamin B₁₂. Exactly why these bacteria should have the potential to grow on such an unnatural duo of nutrients is puzzling.

Hormonal control of spermatogenesis

from R. V. Short

IN reviewing the hormonal control of mammalian spermatogenesis, Steinberger commented that "knowledge of the biochemical parameters of the hormonal action on spermatogenesis is too meagre even for a limited attempt to formulate a working hypothesis" (*Physiol. Rev.*, **51**, 1; 1971). Research so often seems to be at a standstill, that it is refreshing to record a number of important advances in our understanding of spermatogenesis in the last four years.

There is now growing evidence from clinical (Franchimont *et al.*, *J. clin. Endocr.*, **34**, 1003; 1972; De Kretser *et al.*, *J. clin. Endocr.*, **38**, 787; 1974) and laboratory studies (Setchell *et al.*, *J. Endocr.*, **62**, 675; 1974) that the pituitary gland is somehow aware of the number of spermatozoa that the testis is producing. "Inhibin", a substance first postulated by McCulloch in 1932 (*Science*, **76**, 19; 1932), really seems to exist. It is apparently produced by the germinal cells of the testis, and acts centrally to suppress the secretion of follicle stimulating hormone by the pituitary gland. A number of laboratories throughout the world are actively working on the purification and identification of this new testicular hormone, and success seems near at hand.

We are also beginning to get a clearer idea of the role of the Sertoli cells in the control of spermatogenesis. These cells, which line the seminiferous

tubules of the testis, are in intimate association with the germ cell line at all stages of their development, from spermatogonia to spermatozoa. This close proximity suggests that there could be an important functional inter-relationship between germinal and somatic cells, but hitherto we have lacked any biochemical understanding of the nature of this relationship. Thanks to the collaborative work between Drs Hansson (Oslo), Ritzen (Stockholm) and French (Chapel Hill, North Carolina), we can now begin to understand exactly what is happening.

It seems that the Sertoli cell is stimulated by follicle stimulating hormone to secrete an androgen-binding protein with a high affinity for testosterone and its biologically active metabolite, dihydrotestosterone (*Nature new Biol.*, **246**, 56; 1973; *Nature*, **250**, 387; 1974). As Hansson and his colleagues point out in this issue of *Nature* (page 145), testicular androgens are known to be the principal stimulus for spermatogenesis; they seem to be required for almost all the stages of meiotic division (Steinberger, *Physiol. Rev.*, **51**, 1; 1971). In normal circumstances, luteinising hormone secreted by the pituitary gland acts on the interstitial or Leydig cells of the testis to induce testosterone secretion. Though much of this hormone then enters the systemic circulation, the intratubular androgen binding protein may be a vital link in the chain, enabling some hormone to be captured and concentrated within the lumen of the seminiferous tubules, and subsequently transported to the germ cells where it will stimulate meiosis.

It has long been known that it is possible to maintain complete spermatogenesis in hypophysectomised animals if they are placed on testosterone therapy immediately after the operation. But if the testes are allowed to regress following hypophysectomy, testosterone alone will not restore spermatogenesis, and follicle stimulating hormone is required in addition. The present studies suggest that this is because testosterone is only capable of maintaining the secretion of androgen-binding protein by fully developed Sertoli cells.

If the male germ cells make inhibin which regulates follicle stimulating hormone secretion, and if this in turn controls the rate of spermatogenesis by influencing the secretion of androgen-binding protein from the Sertoli cells, we have a subtle biochemical interplay between the germinal and somatic elements of the testis. But what of the ovary? The anatomical relationship between the cumulus cells and the oocyte is reminiscent of that between the Sertoli cells and the spermatogonia. Does the mature oocyte produce the

female equivalent of inhibin, and hence regulate gonadotrophin secretion? Do pituitary gonadotrophins act on the granulosa cells to influence secretions which in turn regulate maturation? Little thought has been given to such problems, and yet they might add a new dimension to our understanding of the complex sequence of endocrine events leading up to ovulation.

What are the parents?

from W. T. Toner

New ideas are needed to explain muon to pion production ratios as large as 10^{-4} in high energy proton interactions. Unlike pions, muons and electrons are not strongly interacting and should not be seen in these collisions, except as rare decay products of strongly interacting parents.

Anomalously high production of large transverse momentum muons and electrons was reported last summer by several groups in the USA, at CERN and in the USSR, working with protons at energies above 70 GeV. Roughly equal amounts of μ^+ , μ^- , e^+ and e^- are observed. In a recent issue of *Physical Review Letters* (**34**, 103; 1975) Leipuner *et al.* claim a similar result for muons with no transverse momentum, in proton-nucleus collisions at only 28 GeV.

It is ironic that this latest result comes from a re-analysis of a 1969 experiment which showed the absence of one hundred times larger amounts of 'direct' muon production, then claimed by a Utah group to take place in cosmic ray interactions. Those claims found no support and were later withdrawn. At that time, there was no compelling reason to make a painstaking analysis of the small residue, which was thought to come from the rare electromagnetic decays of the ρ^0 , ω^0 and ϕ^0 vector mesons, strongly interacting analogues of the photon. But models based on this idea require that most of the pions observed at the same energies and angles as the muons originate in the other decays of these same three particles: the ρ^0 , ω^0 and ϕ^0 have to be singled out from a hundred and one other ways of generating the ubiquitous pion.

Gordon *et al.* (*Phys. Rev. Lett.*, **34**, 284; 1975) suggest that this may happen at large transverse momentum, if the rapid increase that they observe in the yield of ρ^0 relative to pions continues beyond 1 GeV/c transverse. There is an intuitive appeal to such an idea, since the production of all types of particles at large transverse momentum is unexpectedly high and shows features suggestive

of some electromagnetic analogy. How to turn it into a theory is another matter. Besides, another mechanism still has to be found to explain Leipuner's data at low transverse momentum, where vector mesons can only account for 10% of the observed muon yield. The decay of the η meson (a kind of heavy neutral pion) into two muons and a photon might account for another 15% in this case, but would upset the observed balance between electrons and muons if used to explain the large transverse momentum data.

Unfortunately, lack of data on the production of unstable particles prevents us dismissing out of hand the unlikely possibility that known heavier relatives of the ρ^0 , ω^0 and ϕ^0 give rise to the bulk of the muons and electrons. They are included in the generic title 'heavy virtual photons' given to the unknown parents.

An assortment of ordinary mechanisms would not satisfy the discoverers of direct muons, whose searches had a more glamorous stimulus. Theorists have found it necessary to postulate the existence of many new particles in order to account for various features of the weak interaction, more than just the field particles needed to carry the weak force. Those ideas have gained more support with the recent discovery of neutral weak currents.

According to one class of theories, there should be a whole set of new strongly interacting particles with a new quantum number 'charm'. There could even be a fourth degree of freedom, 'colour', to add to isotopic spin, strangeness and charm and give rise to its own family of particles. Strong decays of the new particles would be inhibited by selection rules, giving plenty of opportunity for 'direct' muons and electrons to be produced by weak or electromagnetic decays.

The J and ψ particles may fit such a scheme, but if their production is as infrequent as is rumoured they cannot

be the source of so many direct muons. Are there others, with masses low enough to be produced in 28 GeV proton-uranium collisions?

We can expect many results bearing on these questions in the next few months. A new impetus has been given to attempts to measure the production characteristics of the well known unstable particles. The complete results of Ting's J -particle experiment should answer some of the mundane questions as well as the more exciting ones. Every high energy laboratory is mounting programmes to search for charm and colour. Also, many groups are looking again at their 'zoos' of supposed artefacts, conscious that neutral currents, J -particles and the direct muons all gave signs of their presence in earlier experiments, though too faint to create universal enthusiasm for following them up.

Polyacrylamide gels

from Paul Calvert

CROSS-LINKED polyacrylamide gels are extensively used in the electrophoretic separation of proteins. It is thought that the gel acts as a molecular sieve with a distribution of pore sizes in the 20 Å range so that small molecules pass easily through and large molecules are retarded. These gels are prepared by the free radical polymerisation of an aqueous solution of a few per cent acrylamide containing a small amount of cross-linking agent. Under these conditions one might reasonably expect to get a homogeneous network. Pore sizes calculated on this assumption (see for instance Robard and Chrombach, *Proc. natn. Acad. Sci. U.S.A.*, **65**, 970; 1970; Hersey and Rees, *Nature phys. Sci.*, **230**, 92; 1971) are about right for protein dimensions and thus we have a fine example of a simple theory giving good results.

Unfortunately for lovers of simple theories, recent scanning electron microscopy of freeze dried gels suggests that the structure is not homogeneous at all. Blank and Reimschuessel (*J. Materials Sci.*, **9**, 1815; 1974) studied similar polyacrylamide gels from the point of view of their suitability as support media for crystal growth. Their results show a structure consisting of 2–10 μm walls, surrounding pores of similar dimensions, in a 2.5 weight per cent acrylamide gel. As the concentration is increased the walls become thicker. This size is suitable for the growth of crystals but huge compared to any protein molecule so that if there is a sieving action it must be within the walls, and will depend on the pores not being interconnected.

First we must consider whether the pores are an artefact of the preparation

technique. The gels were prepared by ^{60}Co irradiation rather than by a catalyst-accelerator system but this should not be a crucial difference. The usual concentration of cross-linking agent was used. The freeze drying may have distorted the structure but the kind of large-scale chain breakage needed to create the pores seems unlikely. Further, a cross-linked polyvinyl alcohol gel showed only very small pores after freeze drying.

There have been many reports of anomalous behaviour of organic gels. In gelatin gels Johnson and Thornton (*J. Photog. Sci.*, **16**, 117; 1968) showed that 1 μm particles have considerable mobility. Pines and Prins (*Macromolecules*, **6**, 888; 1973) have demonstrated phase separation in agarose and some polyvinyl alcohol gels but not in gelatin.

Thus there is some evidence for a macroscopic pore structure in several gel systems which must arise by a liquid-liquid phase separation process analogous to that observed in many polymer solutions below the theta temperature (see Flory's *Principles of Polymer Chemistry*) but with the additional constraint of the cross-linked structure to be accounted for. In terms of the electrophoretic separation the presence of the macroscopic pores cannot do much good and it seems that further study of the relationship between structure and separation could lead to improved gel systems.

Changes in species diversity

from Peter D. Moore

IF there is one area of ecology in which theoretical speculation has outpaced acquisition of field data it is in the study of succession. The time scale involved in most terrestrial successions is of the order of centuries, which leaves the hopeful student with limited options: he must either be contented with the study of a series of extant phases which are assumed to be related in a successional series, or he must turn to systems which attain a more rapid equilibrium (such as plankton) for the formulation or confirmation of generalised models. Neither course is satisfactory, but life is short.

The former alternative has recently been chosen by Nicholson and Monk (*Ecology*, **55**, 1075; 1974) as a means of investigating the vexed problem of diversity changes during succession. By obtaining data on the vascular plant composition of 51 seral vegetation types developed on abandoned agricultural land in the Georgia Piedmont, they have produced one of the most extensive sets of information currently avail-



A hundred years ago

A METEOR was not only seen but actually caught at Orleans on the 9th inst. A small mass of pyritous substance was discovered in one of the streets, at the very place which had been struck by an immense flame a few seconds before. The pieces were divided among bystanders anxious to secure the possession of the smallest fragment of such a celestial object; but it is hoped some of the possessors will be intelligent enough to get a specimen sent to the Academy of Sciences.

from *Nature*, **11**, 396; March 18, 1875

able for the study of diversity changes.

The mathematical definition of diversity is in itself a controversial subject. Perhaps the most commonly used and widely accepted index of diversity is that derived from the information theory of Shannon and Weaver, which was first introduced into the ecological arena by Margalef (*Gen. Syst.*, 3, 36; 1958). Although this function has advantages as a general index, it has one major drawback, this being that it compounds species richness (number of species per unit area or in relation to the total number of individuals) and equitability or evenness (the apportionment of individuals among the species present). Both Odum (*Science*, 164, 262; 1969) and Pielou (*An Introduction to Mathematical Ecology*, Wiley, New York) have pointed out that these two parameters may behave differently during succession and that such differences would be obscured by a simple consideration of the Shannon function.

Nicholson and Monk have calculated richness, information content (Shannon diversity) and equitability for their data and in doing so have confirmed Odum's and Pielou's suspicions. Richness and equitability (they use Pielou's index, J , which divides the Shannon index by the natural logarithm of the number of species: see *J. theor. Biol.*, 13, 131; 1966) do behave differently and information content follows an intermediate path. Richness increases rapidly during the first 70 yr of abandonment and then continues to rise gradually over the next 130 yr, but within this general trend one can detect periods of stagnation at 10–20 yr (dominance of perennial grass species), 30–80 yr (pine dominance) and at 150–200 yr (climax species, such as *Fagus grandifolia*, attain dominance). Equitability, on the other hand, rises to fairly high levels within 20 yr and subsequently levels off; there is even a tendency to fall in the older stands, possibly due to the increasing degree of dominance.

Falling diversity in the later stages of succession has also been observed by Loucks (*Am. Zool.*, 10, 17; 1970) in the western Great Lakes region and by Shafi and Yarranton (*Ecology*, 54, 897; 1973) in the boreal forest of northern Ontario. These authors interpret this as a product of random catastrophic disturbances which typify temperate and boreal vegetation types. For example, within much of the boreal Canadian forests burning is likely to take place at intervals of 200 yr or less. Under such conditions, evolutionary pressures might favour 'immature' vegetation types with low equitability in contrast to tropical forest where low perturbation frequency would favour high species richness and equitability.

Nicholson and Monk favour the concept of evolutionary influences upon

ecosystem composition as far as richness is concerned, but they consider the equitability component of diversity to be more directly constrained by limited resource availability. This would account for the early plateau in the equitability curve since, if resources were limiting, population and biomass growth within any stratum of the vegetation would be curtailed and hence equitability stabilised.

From this work it is evident that diversity should not be considered as a single entity when dealing with successions, but should be reduced to its component parts. It is sad that such analyses of diversity invariably, and perhaps inevitably, deal with single taxonomic groups of organisms such as birds, vascular plants, or lizards. Maybe one should be consoled by the results of Murdoch, Evans and Peterson (*Ecology*, 53, 819; 1972) who found positive correlation between evenness and diversity of plants and evenness and diversity of Homoptera within old field successions in Michigan. Perhaps a consideration of plants alone does have some relevance and relationship to the behaviour of the entire biota during the course of succession, but over-generalisation could still be very misleading.

Probing the transcript

from Paul Tolstoshev

THE complementary DNA copy of specific messenger RNA molecules has provided the molecular biologist with a powerful analytical tool. Such cDNA molecules are synthesised from mRNA by viral RNA-dependent DNA polymerase, and can be labelled to very high specific radioactivities. Molecular hybridisation analysis, using such a cDNA probe, allows the detection of extremely small amounts of a specific mRNA sequence.

The analysis, with globin specific cDNA, of RNA transcribed from isolated chromatin by *Escherichia coli* RNA polymerase, has yielded spectacular results. The *E. coli* enzyme seems to transcribe globin-specific sequences from duck reticulocyte chromatin (Axel *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, 70, 2029; 1973), from mouse foetal liver chromatin (Gilmour and Paul, *Proc. natn. Acad. Sci. U.S.A.*, 70, 3440; 1973) and from rabbit bone marrow chromatin (Steggles *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, 71, 1219; 1973), but not from duck liver, rabbit liver, or mouse liver chromatin, or from free duck DNA. Such results imply that isolated chromatin retains some specificity with respect to the genetic sequences which are available for transcription. Furthermore, the

specificity of transcription seems to be mediated by the non-histone protein fraction of the chromatin. This conclusion was derived from the chromatin reconstitution experiments of Paul *et al.* (*Cold Spring Harb. Symp. quant. Biol.*, 38, 885; 1973), and has recently been confirmed by the experiments of Barrett *et al.* (*Proc. natn. Acad. Sci. U.S.A.*, 71, 5057; 1974).

A major problem in assessing the validity of chromatin transcription experiments of this type lies in the presence of endogenous RNA sequences in chromatin preparations. Most chromatin preparations contain RNA, and it is perhaps to be expected that globin-specific sequences are contained in the RNA present in chromatin from erythropoietic tissue. The cDNA probe cannot, of course, distinguish between endogenous and newly synthesised globin sequences. Paul *et al.* labelled the transcribed RNA with ^{32}P . The hybrid between cDNA and transcribed RNA contained sufficient ^{32}P radioactivity to indicate that a large portion of the globin sequences detected had been newly synthesised. But the very small amounts of ^{32}P label in the hybrid make quantitative estimations rather imprecise.

Barrett *et al.* measured amounts of endogenous globin sequences in RNA from native chicken reticulocyte chromatin, and found relatively high levels (about 0.02% of the total endogenous RNA). Transcription of native reticulocyte chromatin by *E. coli* RNA polymerase yielded additional globin-specific sequences in amounts approximately the same as those of the endogenous sequences. But when chromatin was separated into DNA, histone and non-histone components, and was reconstituted, either from chicken erythrocyte DNA and histones, and reticulocyte non-histone components, or from all reticulocyte components, no endogenous globin-specific sequences were detected. Transcription of the reconstituted chromatins yielded RNA containing 0.007 to 0.025% globin-specific sequences. Thus it seems that the preparative procedures used by these authors for DNA, histone, and non-histone protein isolation allowed few or none of the endogenous globin-specific sequences to survive. This was confirmed, for the non-histone protein fraction, in a control experiment where erythrocyte DNA and reticulocyte non-histone protein were reconstituted in the absence of any histone. When this material was transcribed, no globin-specific sequences were detected.

This suggests that there are no globin sequences contaminating the reticulocyte non-histone protein fraction, as well as establishing a histone dependence for correct chromatin

reconstitution. Barrett *et al.* were able to demonstrate that substitution of the reticulocyte non-histone proteins by chicken liver non-histone proteins prevented the transcription of globin sequences. The specificity observed in chromatin reconstitution must therefore be attributed to components of the non-histone protein fraction.

The use of such chromatin reconstitution systems as a functional assay for specific non-histone proteins still however remains a formidable task. This may be simplified somewhat if purified euchromatin fractions can be successfully reconstituted in the same way. (Euchromatin is that fraction of the chromatin thought to be active in transcription *in vivo*.) Several groups have isolated fractions of chromatin which seem to have many of the properties expected for euchromatin. There has however been, to date, no demonstration that the DNA of such fractions is enriched for the gene sequences which are transcribed *in vivo*, in the cell of the chromatin's origin.

An interesting study on the nature of the RNA transcribed *in vitro* from isolated chick myoblast chromatin, by a mammalian RNA polymerase (form B from calf thymus) has recently been reported by Groner *et al.* (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 194; 1975). These authors found that most of the transcribed RNA was of relatively small size (5 to 7S). They demonstrated, by isopycnic gradient centrifugation in CsCl, that a large proportion of this transcribed RNA remained bound to the DNA template. The addition of a polyanion, heparin, to their reaction mixture resulted in an increase in the amount and size of the RNA transcript. The RNA now sedimented as a broad peak (between 5 to 50S) on sucrose density gradients. CsCl density gradient analysis revealed that the larger RNA had apparently been released from the template. Pulse-chase experiments established that the small, DNA-bound transcript produced in the heparin was a precursor of the larger material made when heparin was added.

A similar effect of heparin on the amount and size of RNA transcribed from chick oviduct chromatin by both mammalian and bacterial polymerase had previously been reported by Cox (*Eur. J. Biochem.*, **39**, 49; 1973). In this case the effect was attributed to the inhibitory effect of heparin on nucleases present in the chromatin. Control experiments by Groner *et al.* showed that the chromatin used in their study was sufficiently free of ribonuclease activity to eliminate this factor as the major effect of heparin. Groner *et al.* interpret their results as indicating that the small RNA transcripts made in the absence of heparin are transcribed

from, and remain bound to single-stranded regions of DNA. The addition of heparin induces a structural change in the template, resulting in the unwinding of longer stretches of DNA. This, in turn, allows production of much longer transcripts, which are presumably displaced from the template by reformation of the DNA duplex. As the authors point out, their interpretation is consistent with the model of chromatin structure where the unwinding of DNA to single-stranded regions is required for specific transcription (Crick, *Nature*, **234**, 25; 1971).

Two problems arise in this interpretation of the data. First, heparin can cause displacement of chromosomal proteins from DNA (see for example Cox, *ibid.*) and Groner *et al.* did not determine whether such displacement was caused by the relatively high levels of heparin (2.5 mg ml⁻¹) used in their experiments. Second, a search for single-stranded regions of DNA in chromatin by Levy and Simpson (*Nature new biol.*, **241**, 139; 1973) revealed only very low levels (no more than 0.01% of the DNA). Nevertheless, it may be of interest to apply the approach of Groner *et al.* to the transcription of reconstituted chromatin. The use of specific cDNAs may make possible a demonstration that heparin (or other polyanions) can cause release of the transcriptional specificity of chromatin. Thus one might perhaps obtain transcription of sequences not normally produced in the cells of the chromatin's origin. Whether the action of heparin is mimicking, in some gross way, the action of specific gene regulators in eukaryote cells, remains to be established. It is however clear that the *in vitro* reconstitution and transcription of chromatin will continue to be the focus of a great deal of attention in attempts to unravel the intricacies of transcriptional regulation.

Pyroxene geotherm supports plumes

from Peter J. Smith

IN spite of their importance, temperatures in the Earth's interior remain uncertain because they are impossible to measure directly and difficult to estimate indirectly. Recently, however, Boyd (*Geochim. Cosmochim. Acta*, **37**, 2533; 1973) applied pyroxene geothermometry to ultramafic xenoliths in the kimberlite pipes of Lesotho and derived a temperature-depth curve which could well represent real temperatures in the upper mantle. He used phase equilibria in the enstatite-diopside and enstatite-garnet systems to obtain the equilibration temperature and pressure of xeno-

liths containing the mineral assemblage enstatite+diopside+garnet. The resulting geotherm suggests that down to about 160 km temperature increases with depth in reasonable accord with the extrapolation of the near-surface thermal gradient. But at a depth of about 170 km the temperature indicated by the pyroxene geotherm begins to rise much more rapidly, increasing by several hundred degrees over a depth of a few tens of kilometres.

It is difficult to imagine that this rapid temperature rise represents steady state behaviour. But if it is transient (with respect to the lithosphere), what causes it? Boyd's own suggestion was shear heating, which would also explain why the xenoliths from below 170 km are strongly sheared whereas those from above are unshaped. Unfortunately, this hypothesis fails to explain why the intrusions carrying the xenoliths are localised phenomena. A second possibility would be thermal conductivity variation; but the thermal conductivity would have to decrease by a factor of five, a possible but unlikely change.

Parmentier and Turcotte (*Earth planet. Sci. Lett.*, **24**, 209; 1975) therefore propose that the increase in geothermal gradient reflects a relatively localised distortion of the normal mantle convection pattern caused by a mantle plume. As they envisage it, cold lithosphere moves over a hot rising plume. The extra heat arriving at the base of the lithosphere would normally be expected to rise up through the lithosphere by conduction. But conduction is slow, so that before the heat has penetrated very far, any particular section of lithosphere will have moved away from the plume zone. The geotherm in the lithosphere will thus hardly be affected by the plume, except near the base. In other words, the lithospheric and asthenospheric geotherms, which are different, remain largely independent of each other; and this accounts for the point of inflection at about 170 km (although the small amount of conduction into the lithosphere ensures that the two geotherms merge smoothly). By the same token, the depth at which the temperature begins to rise rapidly is the lithosphere-asthenosphere boundary.

Numerical studies of a model involving a cylindrical plume heated from below give results which are consistent with this view. That is to say, the total geotherm predicted from the model is in good agreement with Boyd's pyroxene geotherm as long as the kimberlite intrusion in the model is located away from the plume axis in the direction from which the lithosphere is approaching. Clearly this agreement may be taken as indirect evidence in favour of the existence of mantle plumes.

articles

Mesozoic apparent polar wander and Atlantic plate tectonics

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New palaeomagnetic data indicate that the North American apparent polar wander curve followed an approximately latitudinal path for most of the Mesozoic. This has important implications concerning the early phases of seafloor spreading in the Atlantic, particularly as Africa seems to have been stationary at that time.

THE North American apparent polar wander (APW) curve for the mid Mesozoic is poorly understood and has been the subject of disagreement for some time. Data have been particularly inconsistent for the Jurassic Period^{1,2}. With relatively few reliable Jurassic determinations it has commonly been thought that the APW curve passed from a reasonably well determined point in the Triassic (see Fig. 1a) to a position which is close to the present axial dipole, and then to a well determined Cretaceous position. In fact, the Mesozoic APW curve does not pass through the present axial dipole, but follows an approximately latitudinal path (present-day coordinates) between the Triassic and Cretaceous pole positions^{3,16}.

The direction of the path of the pole during the Mesozoic has a direct bearing on our understanding of the early

opening of the Atlantic, especially in light of African palaeomagnetic data. Published African palaeopoles for the early Triassic to the early Cretaceous are so similar in their positions as to be indistinguishable from one another at the level of statistical confidence of palaeomagnetic data (Fig. 1b). This consistency indicates that Africa was essentially fixed relative to the magnetic pole for most of the Mesozoic. The apparent motion of the North American pole, therefore, indicates that the entire motion related to the early opening of the North Atlantic (the separation of Africa from North America) must have been taken up by movement of the North American plate.

Before examining the constraints that the newly established North American Mesozoic APW curve places on the opening of the Atlantic, a few points regarding the curve itself need to be examined.

North American Triassic poles used by various workers in the reconstruction of the Atlantic always include a group of late Triassic poles displaced more to the north than the rest of the late Triassic poles. They have been cited⁴ as evidence of rapid polar wander. Obviously, the tight grouping of the African poles refutes this hypothesis.

Poles located at these higher latitudes are all derived from

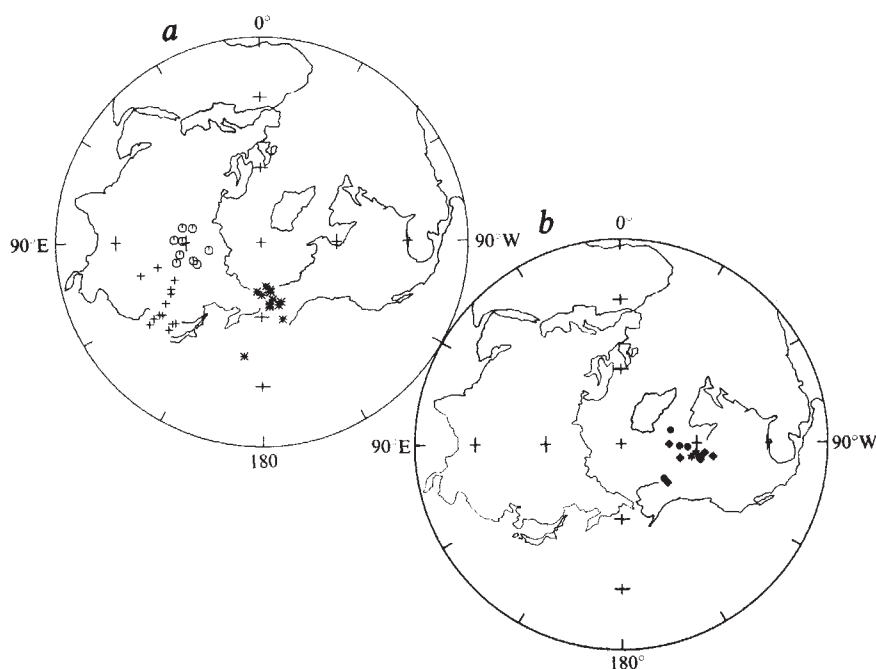


Fig. 1 a, Most reliable¹⁶ published poles for the Permian (+), Triassic (○) and Cretaceous (*), making up the APW curve for North America. b, Most reliable poles (see refs 9 and 15) for the Triassic (●), Jurassic (◆) and Cretaceous (*) of Africa.

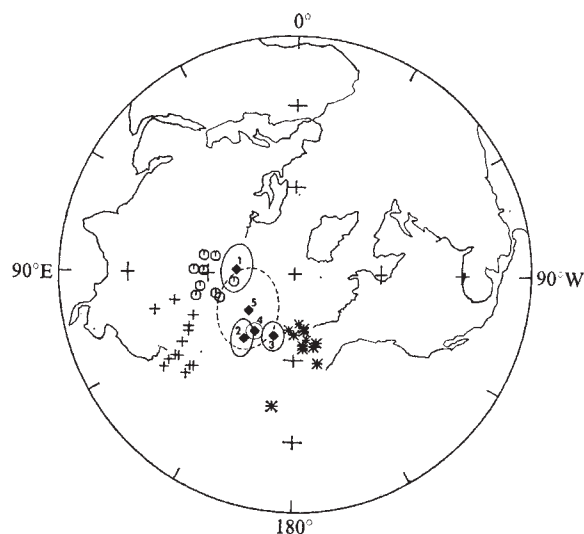


Fig. 2 The North American, Mesozoic APW curve. Poles (♦) and ovals of confidence of: 1, the Summerville Formation¹; 2 and 3, lower and upper Morrison Formation¹⁶; 4, Mesozoic dykes⁶; 5, Topley Intrusions³, against the background of the Permian (crosses), Triassic (open circles) and Cretaceous (*) poles of Fig. 1a.

rocks of the north-eastern USA and have not been observed in late Triassic rocks of western North America. These poles must be viewed with suspicion as they form a streak between the majority of late Triassic poles and the present axial dipole. Armstrong and Besancon³ contend that the Newark Group rocks have been somewhat altered (zeolite facies metamorphism) and are unreliable for K-Ar dating; as most magnetic studies are from rocks associated with the Newark Group it is therefore possible that some of the magnetic directions obtained are unreliable. Regardless of the reason, the likelihood that a real streak would occur in the same direction as that expected from the imprinting of secondary magnetisation is so improbable that it should be questioned. Furthermore, a number of the studies have a very small sample population.

Previous studies on Jurassic rocks have given very inconsistent results. The position of the pole determined from the Upper Jurassic Summerville Formation is, however, not much different from that of Triassic time¹. There are no structural uncertainties associated with the data and remagnetisation of a younger unit into an older direction is impossible. It is unlikely that the data record an odd direction resulting from secular variation as they encompass a reversal of the field, and therefore probably span enough time to average out extremes of secular variation. The Oxfordian Summerville direction seems to indicate that the pole position for North America remained relatively constant from late Triassic to late Jurassic time and that it subsequently moved rather quickly to the Cretaceous position.

Magnetic directions obtained from the unit directly overlying the Summerville, the Morrison Formation¹⁶ are in complete accord with the hypothesis that most Mesozoic APW relative to North America occurred over a relatively short time during the late Jurassic. Two different directions have been obtained from sediments in the lower and upper parts of the Morrison formation (Kimmeridgian-Tithonian respectively). The directions lie in sequential position on the APW curve and suggest that about 10° of apparent motion of the pole occurred during Morrison time. These directions also imply that about twice that amount of apparent motion occurred between the Summerville and the lowest sampled portion of the Morrison (Fig. 2).

(More than 35 m of unsampled sediments, and a probable unconformity, exist between the lowest sampled beds of the Morrison and the Summerville.) The Morrison and Summerville data indicate that the majority of the movement occurred in post-Summerville time, that is, late Oxfordian(?), Kimmeridgian and Tithonian. The oldest dated Cretaceous rocks studied palaeomagnetically show pole positions already located in the position shown by later Cretaceous rocks.

The Morrison data also confirm that the APW path was approximately latitudinal relative to the present axial pole and did not include the present dipole. The average direction of the Topley Intrusions³ agrees with this hypothesis, although the confidence limits on the data are quite large. A pole in the same area has also been obtained by DeBoer⁶ from some dykes of unknown age cutting Upper Triassic rocks.

This APW path requires that the pattern of separation of Africa and North America be somewhat different from that proposed previously. Pitman and Talwani⁷ have computed finite difference poles to describe the rotations needed to open the central Atlantic. Rotation of the North American plate about the oldest (180–155 Myr) pole of opening will produce very little apparent motion of the magnetic pole relative to North America as the magnetic and rotational poles are very close together. That supports my finding¹ that the magnetic pole was approximately static relative to North America from the late Triassic through the mid Jurassic and into the beginning of late Jurassic time.

Rotation about Pitman and Talwani's⁷ next pole of opening (155–81 Myr ago), however, does not move average, late Triassic poles^{8,9} through the positions represented by the Morrison poles. Nor does this rotation move the pole to the position of the North American Cretaceous poles. The positions of the lower Morrison, Topley, and perhaps the Mesozoic dyke poles suggest a two stage rotation during the Jurassic: one additional rotation to that suggested by Pitman and Talwani⁷ for the time between rifting and 81 Myr BP. The pole to this rotation must lie on a great circle that passes midway between the late Triassic and lower Morrison poles. The rotational pole must lie at some point between the small circle connecting these magnetic poles and a point on the North American continent, in order to produce both the APW observed and the actual motion of North America relative to Africa. This indicates that North America must have undergone a clockwise rotation relative to Africa before it moved away causing the opening of the Atlantic. The rotation following the clockwise rotation was probably about Pitman and Talwani's⁷ 180–155 Myr pole of opening, as that moves the pole approximately from the lower Morrison position through the upper Morrison position and into the Cretaceous position.

The path of polar motion defined by the sequential Summerville and Morrison poles corresponds to a change in direction of APW during the Jurassic (Fig. 2). The timing of this change of direction is interesting. The Morrison polarity sequence can be correlated¹⁶ with the oldest anomalies of an M sequence anomaly pattern¹⁷. Thus, the lower Morrison pole represents the pole of the oldest of the Mesozoic anomalies. This means that the change in direction of the pole path occurred just before the formation of the lineated anomalies. The change coincides with the changes from rough to smooth and from magnetically quiet to lineated-anomaly sea floor, and may, therefore, indicate a causal relationship.

The stationary position of Africa throughout most of the Mesozoic may have been conducive to asymmetrical spreading in the Atlantic. Indeed, the quiet zone boundaries on either side of the Atlantic are not symmetrical with respect to the continental margins^{10,11}. At least two mechanisms could produce these different widths of sea floor on either side of the Mid-Atlantic Ridge¹²: either an eastward jump

of the ridge crest or a migrating ridge crest, would produce asymmetry. The stationary position of the African plate requires that the spreading crest be in motion at the time of formation of the marginal quiet zone and the late Jurassic-early Cretaceous anomalies. These data therefore favour the migrating ridge system as the cause of the asymmetry, suggesting that both the North American plate and the ridge crest may have been migrating away from Africa, with the plate moving much faster. Later, however, during the time between the end of the quiet zone and anomaly 32, the African pole still shows no motion although the zones of the sea floor representing this time are quite similar in width on both sides of the Atlantic. This suggests that the ridge crest may by then have started to migrate rapidly enough to produce symmetrical spreading during that time.

If the Morrison and Summerville poles are truly representative of sequential positions of the Jurassic pole along an APW path created by seafloor spreading, very high rates of seafloor motion are implied. An approximate idea of the rates involved can be obtained from equations used by Deutch¹³. Although Pitman and Talwani's⁷ rotational pole does not rotate the mean late Triassic pole into the position of the mean Cretaceous pole, an approximate seafloor spreading rate can be obtained by computing the rate of plate movement about this rotational pole that would bring the Triassic pole closest to the Cretaceous pole. The amount of time allowable for the rotation is considered to be approximately 20 Myr (early Oxfordian to early Cretaceous—for numerical ages see ref. 14), based on the position of the Summerville pole. I have computed the rates at 45° away from the rotation pole, that is, within the area of the sea floor between Africa and North America. A rate of 16–19 cm yr⁻¹ is necessary at this distance to produce the

total opening for that time⁷. (The uncertainty stems from the choice of the various^{1,8,9} Triassic poles which can be used.) The rate for just the motion between early Morrison and early Cretaceous time (around this same rotational pole), allowing 15 Myr (from the base of the Kimmeridgian to the base of the Cretaceous), at 45° from the pole, would be 12.5 cm yr⁻¹. These are quoted as whole rates, as the ridge system was probably also in motion. They are rates for the time of formation of some portion of the quiet zone and anomalies M22 to M25.

Even if the rates are considered in terms of half rates, both computations give a surprisingly high rate of seafloor spreading. The rates are far in excess of any that have been proposed for the Atlantic, and rank among the highest rates ever proposed for any spreading ridge. Such high rates may have occurred as some consequence of the initial spreading process, or they may indicate events that caused a drastic slow-down in Atlantic spreading subsequent to opening.

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Nuclear envelope permeability

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The permeability of the amphibian oocyte nuclear envelope in situ has been determined for three tritiated dextrans. The envelope is a sieve, restricting molecular movement between the cytoplasm and nucleus. The patent radius of its pores is about 45 Å.

MANY substances are known to be in continuous exchange across the nuclear envelope. These include ions and metabolites^{1–3}; histone and non-histone chromosomal proteins^{4–6}; ribosomal and messenger ribonucleoproteins^{7–9}; hormones, either free or bound to protein receptors¹⁰; viral proteins and nucleic acids^{11–13}; and less characterised protein and RNA species^{14–15}. Yet, because quantitative flux data are almost non-existent, we know little about the factors that govern the passage of these substances through the nuclear envelope, and we cannot assess the envelope's role in controlling nucleocytoplasmic exchange.

The most direct method of obtaining rate data on nuclear envelope permeation is to microinject a substance into the cytoplasm and follow its passage into the nucleus with quantitative optical or electron microscopic techniques. Solutes used for such studies have been chosen primarily for technical rather than heuristic reasons. With the advent of ultra-low temperature autoradiography (ULTA)¹⁶, which can quantitatively locate any tracer substance regardless of its diffusibility, a systematic approach to the problem of envelope permeation becomes possible, since any radioactively labelled substance can serve as a probe.

Size has been the most studied of the permeant properties

which influence nuclear envelope passage. Colloidal gold particles greater than 125–145 Å in diameter do not penetrate the nuclear envelope of *Amoeba proteus*¹⁷. Ferritin, with a molecular diameter of 95 Å, does not enter the nuclei of amphibian and cockroach oocytes^{18,19}; below this limit, permeation is inversely related to tracer size. Passage of proteins (molecular weight 12,000–67,000; radii ≈ 15–35 Å) is slowed by the nuclear envelopes of the cockroach oocyte¹⁹ and chironomid salivary gland cell (P.L.P., unpublished), the degree of slowing increasing with increased molecular size. Slowing is not observed when tracers are 4,200 dalton or less.

While these observations reveal some nuclear envelope sieving properties, a more quantitative understanding requires nucleocytoplasmic flux and equilibrium data on a size-graded series of molecules with otherwise similar properties. We have obtained such data for size-characterised fractions of dextran, a polymer of D-glucopyranose units linked by α-1:6, α-1:4, and α-1:3 glucosidic bonds. Dextrans in aqueous solution are considerably hydrated and behave as spheres²⁰. The hydrodynamic radii of our tracer fractions were 12.0, 23.3 and 35.5 Å (Table 1). Solutions of purified ³H-dextran were microinjected into the cytoplasm of mature oocytes of *Rana pipiens*. Solute diffusion was permitted until the cells were quenched at –190 °C. The oocytes were sectioned at –50 °C, and the local intracellular concentrations of tracer determined using ultra-low temperature autoradiography. Figures 1 and 2a and b summarise the preparation and microinjection of the ³H-dextran fractions, the autoradiographic procedures, and the tests for intracellular ³H-dextran degradation.

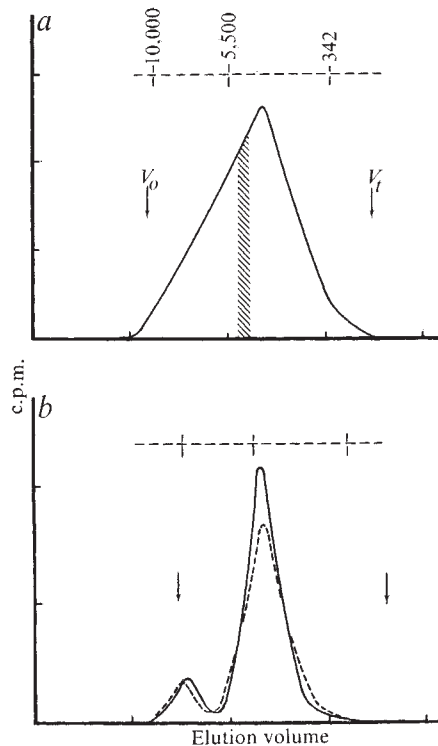


Fig. 1 *a*, Gel chromatographic isolation of 12.0 Å ^3H -dextran tracer. Commercially obtained ^3H -dextran (specific activity 0.4 mCi mg^{-1} , lot No. 622-215, New England Nuclear, Boston) in water was fractionated on Sephadex G-50 to remove $> 10,000$ dalton dextrans (not shown). A portion of the remaining material was rechromatographed on Sephadex G-50, yielding the distribution shown. A subfraction (shaded), with hydrodynamic radius 12.0 Å, was isolated for injection and was concentrated by lyophilisation to 650 $\mu\text{Ci ml}^{-1}$. *b*, The size distribution of the injectate was confirmed on Sephadex G-50 at the time of microinjection (—); no breakdown and only negligible polymerisation (a time-dependent process observed with 12.0 Å dextrans) was detected. Intracellular stability was established by homogenising four cells 40 h after microinjection and chromatographing the 12,000g supernatant (....). ^3H -dextran injectates (23.3 and 35.5 Å) were prepared similarly using Sephadex G-50 and G-75 columns. All columns were equilibrated with water and eluted to stable total volumes (V_t) before use. Void volumes (V_0) were determined with Blue Dextran 2,000, and molecular size calibrations were carried out using sucrose (342), inulin (5,500) and dextrans (10,000, 20,000, 40,000 and 70,000) as standards.

Cytoplasmic diffusion

Immediately after microinjection, the ^3H -dextran is found at the injection site, observed in autoradiographs as an intense concentration of silver grains. The tracer then diffuses radially from the injection site, and grain density transects show time-dependent diffusion profiles. Cytoplasmic diffusion coefficients, D_c , are estimated by comparing the cytoplasmic profile at a known diffusion time (t_d) with theoretical diffusion profiles (Fig. 2c). The analysis has been outlined previously for sucrose²⁶ and inulin²⁷ in the oocyte, for which D_c are 2×10^{-6} and $3 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$, respectively. Our estimates for D_c of 12.0, 23.3, and 35.5 Å ^3H -dextrans are 3.5×10^{-7} , 2.5×10^{-7} and $1.5 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$, respectively. Over the 70-fold molecular weight range of substances studied in the oocyte, D_c are in the range $0.1D_w - 0.4D_w$ (D_w is the solute diffusion coefficient in pure water); this agrees with estimates of D_c/D_w reported for a variety of tracers in other cells^{26,28}.

Nuclear envelope permeation

Grain density profiles of oocytes in which the diffusion front has reached the nucleus reveal the nuclear envelope's influence on solute movement. Figure 3a-d, showing profiles for 12.0 Å ^3H -dextran at $t_d = 3, 5$ and 16 min, and 16 h, are transects made close to the injection site and through the nucleus.

At diffusion equilibrium, 12.0 Å ^3H -dextran is uniformly distributed within both the nucleus and the cytoplasm (Fig. 3d), but it is more concentrated in the nucleus (see also Fig. 4a). A higher concentration in the nucleus than in the cytoplasm has been observed with every solute we have studied in the oocyte. This phenomenon has also been found in other cells and has been attributed to solute exclusion by the cytoplasm, a process widely associated with macromolecular gels²⁷.

At shorter diffusion times, 12.0 Å ^3H -dextran gradients are discontinuous, decreasing sharply at the level of the nuclear envelope (Fig. 3a-b). Since this is obviously not caused by low nuclear solubility, the nuclear envelope must be a substantially greater transport barrier than is a similar thickness of cytoplasm. Intracellular 12.0 Å gradients mirror those in the adjacent cytoplasm. The envelope, although a sufficient barrier to cause gradient discontinuities, is insufficient to isolate nuclear from cytoplasmic gradients. This suggests that the time required for 12.0 Å ^3H -dextran to cross the nuclear envelope is less than that required to diffuse the radial distance of the nucleus.

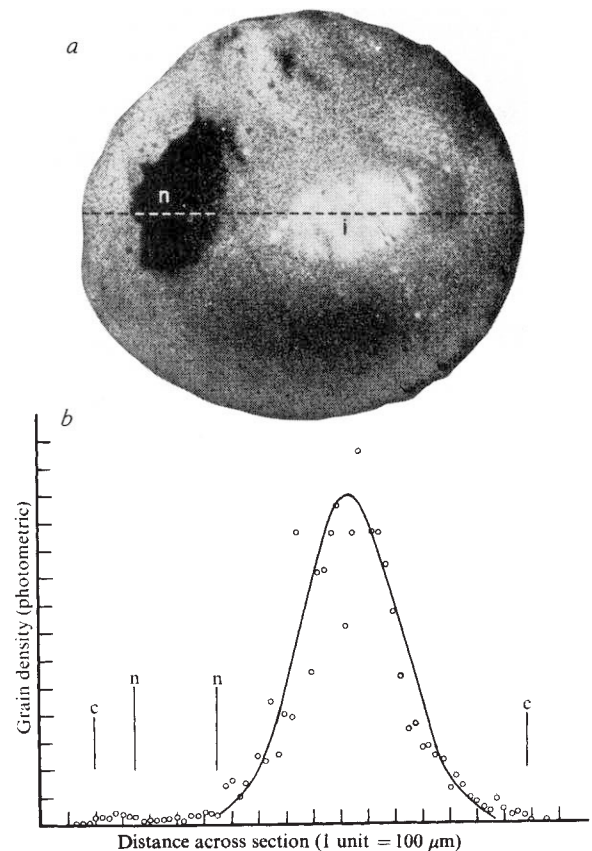


Fig. 2 Microinjection was directed into the vegetal cytoplasm to eliminate the possibility of nuclear damage. Injectate concentrations were 1.6, 1.0 and 0.7 mg ml^{-1} for 12.0, 23.3 and 35.5 Å ^3H -dextrans, respectively; and volumes were $5 \times 10^{-5} \text{ ml}$, or about 2% that of the cell. After a period of time (t_d), the oocyte was rapidly embedded in OCT and quenched to -190°C in liquid nitrogen. Sectioning and autoradiographic exposure were carried out at -50°C and -96°C , respectively. This procedure prevents solute redistribution from the time of freezing until completion of the autoradiograph¹⁶. *a*, Autoradiograph, in darkfield illumination, of a section through both the injection site (i) and nucleus (n) of an oocyte injected with 12.0 Å ^3H -dextran ($t_d = 8 \text{ min}$). Grain counting was carried out visually, in phase contrast at $\times 2,000$, or photometrically, with a Leitz MPV-1 microscope photometer system employing Ploem incident-light illumination²⁴. Grain density values throughout have been corrected for the difference in nuclear and cytoplasmic water content²⁵ and are proportional to ^3H -dextran concentrations on a water basis. *b*, Grain density profile taken along the axis shown as a dotted line in (a). The vertical bars, c and n, mark the cell and nuclear boundaries. The solid line is the theoretical diffusion gradient for $D_c = 3.5 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$, assuming that the injection volume is small enough to constitute an instantaneous point source, and that solute reflection from the cell boundaries is negligible.

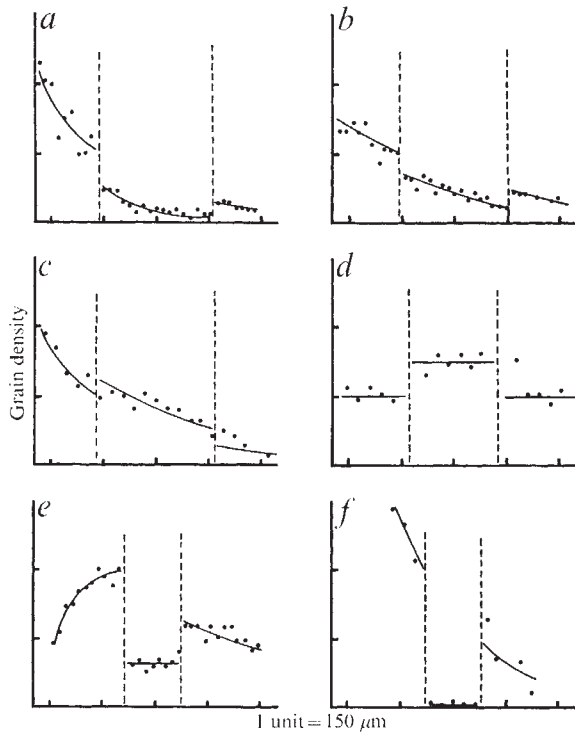


Fig. 3 Grain density profiles through the nucleus (indicated by vertical dashed lines) and adjacent cytoplasm of ^3H -dextran injected oocytes. *a-d*, 12.0 Å, $t_d = 3$ min, 5 min, 16 min, and 16 h, respectively; *e*, 23.3 Å, $t_d = 10$ min; *f*, 35.5 Å, $t_d = 42$ min.

Figure 3*e* and *f* are grain density profiles for 23.3 and 35.5 Å ^3H -dextrans. Both profiles decrease more sharply at the nuclear envelope than do the 12.0 Å ^3H -dextran profiles at comparable times, but, unlike the latter, the 23.3 and 35.5 Å profiles show uniform transnuclear concentrations, in spite of marked gradients in the adjacent cytoplasm. These profiles demonstrate that the nuclear envelope is less permeable to larger dextrans than to smaller, and that envelope permeability can play a major role in limiting the rate of nuclear entry.

Quantitative expression of the envelope's influence on solute transport is complicated by the fact that cytoplasmic and nucleoplasmic diffusions are not instantaneous; hence, each point on the nuclear envelope experiences different and changing concentrations of the permeating solute. To deal with this problem, we compared solute concentrations in adjacent nuclear and cytoplasmic positions by determining the grain density, G , at Q positions ($i, j, k, \dots$) inside, but close to the edge of, the nucleus ($G_n^i, G_n^j, \dots$) and in the adjacent cytoplasm ($G_c^i, G_c^j, \dots$). The mean of the ratios of adjacent nuclear and cytoplasmic sites is the nuclear:cytoplasmic grain density, given by

$$X_{n/c} = (G_n^i/G_c^i + G_n^j/G_c^j + \dots)/Q \quad (1)$$

$X_{n/c}$, expressed as a function of t_d , is a measure of the time course of nuclear envelope permeation. Figure 4*a* shows this for the three ^3H -dextrans investigated. The nuclear entry rates are markedly different; equilibrium is reached for 12.0 and 23.3 Å ^3H -dextrans in about 0.5 and 15 h, respectively, with an equilibrium distribution ratio, $K_{n/c}$, of 1.45. The 35.5 Å ^3H -dextran enters much more slowly, $X_{n/c}$ at 23 h being 0.1, and does not achieve equilibrium during the experiments.

If cytoplasmic and nuclear diffusion are rapid as compared with envelope permeation, and if permeant dwell time within the envelope is short relative to t_d (as the profiles of Fig. 3 suggest), the kinetics of diffusional nuclear filling can be described by

$$1 - (X_{n/c}/K_{n/c}) = \exp(-k_n t_d) \quad (2)$$

in which the rate constant, k_n , is given by

$$k_n = P_n A/V_n \quad (3)$$

where P_n is the nuclear envelope permeability coefficient, A is the envelope area available for permeation, and V_n is the nuclear volume. If cytoplasmic or nucleoplasmic diffusion significantly affected nuclear entry, a higher order equation would be necessary to describe the kinetics. The greater such intra-compartmental diffusion influences, the more poorly equation (2) will describe nucleocytoplasmic tracer movement²⁸.

The nuclear entry kinetics of all three ^3H -dextrans are described by first order exponential equations of the form of equation (2), (Fig. 4*b-d*). We conclude that $X_{n/c}$ corrects for spatial differences in tracer concentration arising from the transcellular diffusion, and that nuclear entry is primarily restricted by the nuclear envelope. The experimentally determined rate constants, k_n , are given in Table 1. Although the 35.5 Å ^3H -dextran differs in molecular radius from the 12.0 Å by a factor of only 2.9 its rate of permeation differs by a factor of more than 2,500. This sieving effect seems to arise from the specialised porous structure of the nuclear envelope.

Functional pore size

If we view the nuclear envelope as a uniformly permeable barrier, A in equation (3) is the total nuclear surface area and, assuming a spherical nucleus of radius r_n , we have

$$P_n = k_n r_n/3 \quad (4)$$

Fig. 4 *a*, Time course of nuclear envelope permeation, expressed as the average nuclear:cytoplasmic grain density, $\bar{X}_{n/c}$ (see text), as a function of diffusion time after microinjection, t_d , for 12.0 (●), 23.3 (○), and 35.5 Å (△) ^3H -dextrans. Vertical bars indicate s.e. mean. *b-d*, First order exponential entry kinetics illustrated by plots of $\ln(1 - \bar{X}_{n/c}/K_{n/c})$ as a function of t_d , for 12.0, 23.3 and 35.5 Å ^3H -dextrans, respectively. $K_{n/c}$ for 35.5 Å ^3H -dextran is assumed to be 1.45, the same value observed for 12.0 and 23.3 Å ^3H -dextrans. Note the different time scales on the abscissae. The rate constants, k_n of Table 1 are given by the slopes of the linear least squares regression lines (constrained to zero on the ordinate) fitting the data points.

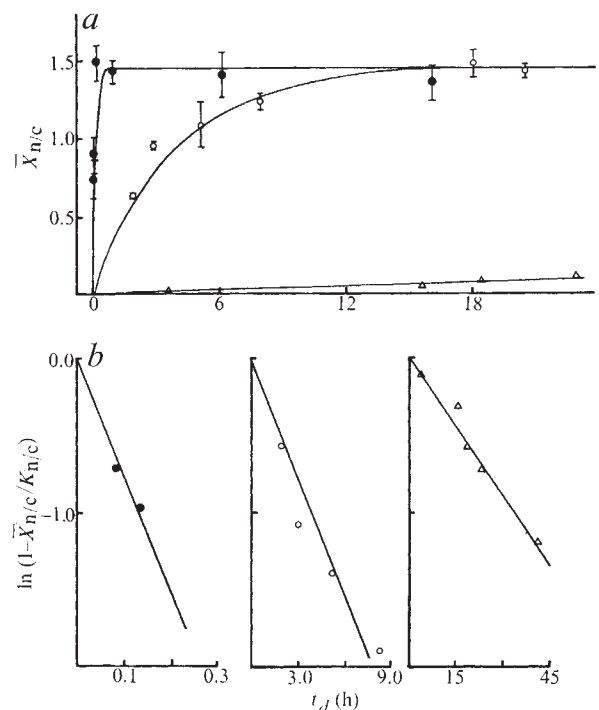


Table 1 ^3H -dextran tracer data

Radius (Å)	$k_n^\dagger$ (s $^{-1}$)	P_n (cm s $^{-1}$)	A_p (cm 2)
12.0*	$(2.13 \pm 2.94) \times 10^{-3}$	1.60×10^{-5}	2.21×10^{-13}
23.3†	$(7.16 \pm 2.61) \times 10^{-5}$	5.37×10^{-7}	1.04×10^{-14}
35.5†	$(8.31 \pm 1.66) \times 10^{-7}$	6.23×10^{-9}	2.01×10^{-16}

*Determined from position of elution peak from Sephadex G-50 ($K_{av} = 0.40$) and the calculations of Laurent and Killander²¹.

†Weight average molecular weights (M_w) were determined by Sephadex chromatography with standard dextrans, and the corresponding molecular radii taken from the measurements of Granath and Kvist²².

‡The k_n is the slope of the least squares regression line calculated to fit the data points and constrained to zero on the ordinate (Fig. 4b-d). The $\pm$ value given is the 95% confidence interval for the slope²³; the confidence interval for 12.0 Å dextran is misleadingly large since only two pre-equilibration data points were obtained, and the method of calculation does not admit use of the relevant early equilibrium points shown in Fig. 4a.

P_n and A_p calculated as explained in text ($r_n = 225 \mu\text{m}$).

from which we can calculate the envelope permeability coefficient, P_n , for each ^3H -dextran (Table 1). P_n is probably not, however, a functionally significant measure of nuclear membrane properties. There is reason to believe, from both envelope ultrastructure and electron microscopic studies of colloidal gold and *in vivo* ribonucleoproteins, that passage of macromolecules through the envelope is restricted to a cylindrical central channel of each nuclear pore complex³. If this is true for our ^3H -dextran tracers, we can calculate the total effective envelope pore area available for permeation, A_p^{total} , substituting D_p/d for P_n in equation (3) where D_p is the ^3H -dextran diffusion coefficient within the pores, and d is the pore complex length. Then

$$A_p^{\text{total}} = k_n V_n d / D_p \quad (5)$$

and the mean effective area available for diffusion through each pore, A_p , is given by

$$A_p = A_p^{\text{total}} / N \quad (6)$$

where N is the number of patent pores per nucleus. The best available estimate of d is 1,500 Å (refs 29–33), and of N , 1.97×10^7 (refs 34 and 35). D_p is assumed to be equal to D_c , and A_p for each ^3H -dextran is shown in Table 1.

For a membrane with pores of radius r , A_p is equal to the total patent area of a pore, πr^2 , reduced by two solute-pore interaction factors, each a function of a/r (refs 36 and 37). The first expresses the steric hindrance to pore entry³⁸; the second is a 'wall correction factor', K_1 , describing the frictional resistance to diffusion within a cylindrical pore relative to that in free solution. Thus, A_p decreases with increasing tracer radius a :

$$A_p = \pi r^2 [1 - (a/r)]^2 (1/K_1) = \pi (r - a)^2 / K_1 \quad (7)$$

Haberman and Sayre³⁹ provided a theoretical solution for K_1 which fits well the available experimental results over a large range of a/r (ref. 40). We have applied computer techniques to the solution method of Haberman and Sayre to calculate the values of K_1 over the range $0 < a/r < 1.0$ at intervals of 0.02 units (P.L.P., and P. Scherr, unpublished). The calculated K_1 values were used to construct the curves in Fig. 5, which illustrates the dependence of A_p on a and r . The experimentally determined A_p for 12.0, 23.3, and 35.5 Å dextrans correspond to r values, determined from equation (7), of 49.0, 40.7, and 43.3 Å, respectively. The mean of these determinations is 44.3 Å; in Fig. 5, the three plotted A_p values are best fit by the $r = 45$ Å curve. We conclude that the ^3H -dextran nuclear entry kinetics can be explained quantitatively as restricted diffusion through nuclear pores with patent radii of about 45 Å.

There is some uncertainty over the factors (V_n , d , D_p , and N) which enter the calculations of A_p . We have determined the ranges

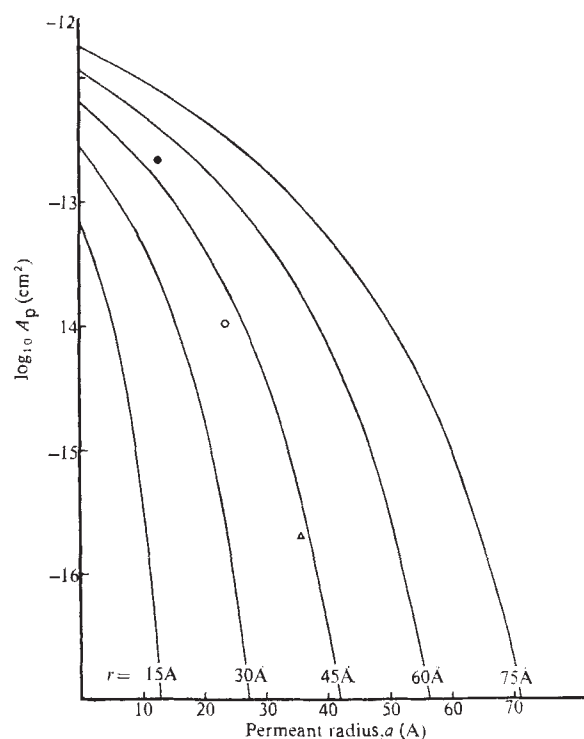
of A_p , and the corresponding ranges of r , resulting from reasonable ranges of each factor around the values used above. For the 35.5 Å ^3H -dextran data, an error of $\pm 10\%$ in the value of the nuclear radius (r_n) would correspond to a range of less than ± 1 Å about the determined r . Possible ranges for the values of D_p (0.1 – $0.4 \times D_w$), d (750–2,000 Å), (refs 30, 35 and 41), and N (1.02 – 3.12×10^7), (refs 34, 35 and 42) each correspond to ranges less than ± 3 Å about the 35.5 Å ^3H -dextran determined value of r . Similarly, variation of $\pm 10\%$ in the value used for 35.5 Å ^3H -dextran radius (a), results in a ± 4 Å change in r . Thus, unless all values chosen err in the same direction, determination of r from A_p and a by equation (7) is not greatly affected by possible errors in the values of the factors used.

Permeability of the mature oocyte nuclear envelope

Results of earlier studies of the permeability of the mature amphibian oocyte nuclear envelope are consistent with the nuclear entry kinetics expected for passage through an envelope with pores of radius 45 Å, as reported here. The solutes used, in order of increasing size, include sodium¹, glycerol⁴³, cysteamine⁴⁴, sucrose²⁶, inulin²⁷, histones¹⁸, polyvinylpyrrolidone-coated colloidal gold^{45,46}, bovine serum albumin (BSA), (ref. 18), and ferritin¹⁸. Solute size of sucrose ($a = 4.4$ Å, ref. 36) or smaller reportedly enter the oocyte nucleus rapidly. Intermediate size solutes—histones, colloidal gold, and BSA—enter more slowly, while ferritin ($a = 47$ Å) does not enter at all.

In principle, diffusion of all solutes is influenced by the nuclear envelope. Our ability to detect this influence, however, is limited to cases in which half-times for nucleocytoplasmic equilibrium, $t_{1/2}$, are at least several minutes—the time required to microinject and freeze the oocyte. In practice this condition is met only with larger solutes, as illustrated in Fig. 6a, where $t_{1/2}$ for the mature oocyte nucleus at 15 °C is plotted as a function of permeant radius, a . As examples, in the oocyte with

Fig. 5 Mean effective cross-sectional area for diffusion through each pore (A_p), as defined by equation (7), plotted as a function of tracer radius, a , for several pore radii, r . Values of A_p determined experimentally (equations (5) and (6)) for 12.0 (●), 23.3 (○) and 35.5 Å (Δ) ^3H -dextrans are indicated by points, and are best fit by the $r = 45$ Å curve.



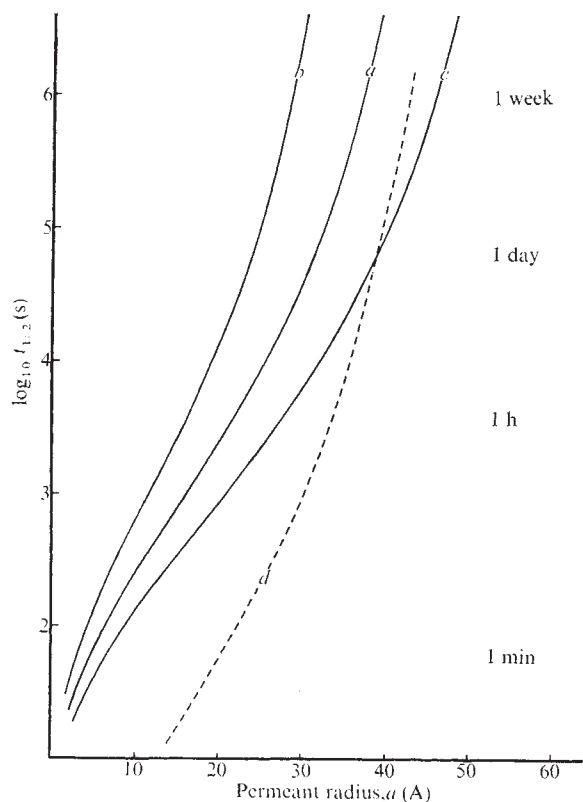


Fig. 6 Half-times for nucleocytoplasmic equilibration of permeants with radius a by restricted diffusion through nuclear envelope pores (at 15 °C). a , Mature amphibian oocyte nucleus, with $r = 45$ Å; b and c , oocyte nucleus, if $r = 35$ and 55 Å, respectively; d , cell with $5\text{ }\mu\text{m}$ nuclear radius and $r = 45$ Å.

$r = 45$ Å a solute with radius 5 Å exchanges with a $t_{1/2}$ of about 1 min, and the reported²⁷ inability to detect an envelope influence on oocyte nuclear entry of inulin ($a = 14.8$ Å, ref. 22, $t_{1/2} \approx 10$ min) probably results from the use of too large a value for t_d as the earliest time studied.

Since $t_{1/2}$ is proportional to nuclear radius (equations (2) and (3)), the entry half-time for a given permeant would be shorter in smaller cells than in the oocyte. Fig. 6d is a plot of $t_{1/2}$ against a for a $5\text{ }\mu\text{m}$ radius nucleus assumed to share all of the properties (other than size) of the oocyte nucleus. The plot provides a notion of the time scale of nuclear permeation to be expected in the typically smaller cells of most multicellular organism tissues.

Nuclear envelope permeability in other eukaryotes

Generalisations based on the present analysis will be valid only to the extent that the amphibian oocyte nuclear envelope is similar to other eukaryote envelopes. Nuclear envelope ultrastructure is extraordinarily conservative; envelope similarity has been demonstrated in cells as phylogenetically distant as amphibian oocytes, amoebae, dipteran salivary gland cells, HeLa cells, normal and cancerous liver cells, and onion root tip cells^{3,33,47}. Several studies using insect oocytes^{19,48}, amoebae⁴⁹, HeLa cells⁶, and salivary gland cells (P.L.P., unpublished), have yielded semi-quantitative nucleocytoplasmic exchange kinetics consistent with a patent pore radius about that determined in the present quantitative experiment. These studies included microinjection of fluorescein-labelled proteins, electron microscopic localisation of colloidal gold and ferritin, and autoradiographic localisation of endogenous proteins. Thus, both morphological and tracer studies suggest that the mature amphibian oocyte nuclear envelope is representative of eukaryote envelopes in general.

The eukaryotic envelope consists of two 70–80 Å unit membranes, separated by a perinuclear space, and fused at intervals to form pore complexes 400–1,000 Å in diameter. An element of each complex is a cylindrical proteinaceous structure extending through the complex into both cytoplasm and nucleoplasm³. These structures are annular in cross section and are widely considered to be tubes with patent central channels of 50–300 Å, most frequently 100–150 Å (refs 32, 34 and 50). Three lines of evidence support the view that these pore-complex channels are the sites of nucleocytoplasmic macromolecular exchange: (1) Colloidal gold particles, passing from cytoplasm to nucleus, seem to be limited, by the annular material, to these central channels¹⁷; (2) ribonucleoprotein-containing particles in nucleocytoplasmic transit seem to narrow down to 100–200 Å in diameter as they squeeze through the central region of the pore complex⁴⁹; and (3) as shown here, a model based on restricted diffusion through tubular pores could account for a 2,500-fold difference in the rate of nuclear envelope permeation among solutes which differ only in molecular size. If this model of the nuclear envelope as a diffusion-restricting barrier with patent pore radii of about 45 Å can be extended to include other cells, significant implications follow.

Diffusible cellular metabolites and proteins with radii smaller than 45 Å move between nucleus and cytoplasm through pores at rates influenced by solute size (that is, a/r). As illustrated by plots of $t_{1/2}$ against a (Fig. 6), relatively small changes in the effective radius of the solute profoundly affect rates of envelope permeation, thus providing the cell with a method of controlling the nucleocytoplasmic movement of solutes. In this regard, there is evidence to suggest that the ability of cytoplasmic oestrogen receptor protein to enter the nucleus is controlled by enzymatic modification of the receptor's dimensions^{10,51}.

Most cellular proteins are of a size⁵² that makes their movement susceptible to influence by small changes in pore radius. Hence, it is possible that the envelope controls nucleocytoplasmic movement by variation of the patent pore radius. Indeed, there is evidence that the patent pore radius is different in different cell types and varies within a single cell type as a function of the cell cycle and nutritional state³. Comparison of Fig. 6a–c shows that a ± 10 Å change in the oocyte pore radius would effect a two- to threefold change in the $t_{1/2}$ of ions and metabolites ($a = 5$ Å), a tenfold change in the $t_{1/2}$ of small proteins ($a = 18$ Å), and more than a 1,000-fold change in the $t_{1/2}$ of large proteins ($a > 31$ Å).

Among the most important materials transported between nucleus and cytoplasm are ribonucleoprotein particles, which are believed to be the transport forms of mRNA and rRNA. These have radii greater than 45 Å and are too large to diffuse through the nuclear pores. Conformational changes in the particles and/or the pore complexes would be necessary for their transport. Such changes have been inferred from electron micrographs^{53–56} and suggest the possibility of nuclear pore complex control of protein synthesis.

Finally, as solute size approaches the dimensions of the pore, solute–pore wall interactions become increasingly important. Specific site interactions, for example H-bonding and charge–charge interactions, would also then influence solute movements. The demonstration that *Rana pipiens* annular material proteins have a net positive charge⁴⁶ supports the idea that factors other than size influence nuclear envelope permeability.

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Sequence of the O_R operator of phage λ

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A sequence of 79 nucleotides from the λ O_R operator is obtained by primed transcription of repressor protected DNA fragments. The sequence contains the primary repressor binding site plus partial duplications which can be interpreted as secondary repressor binding sites.

THE λ repressor is a protein which controls the functioning of early phage genes by binding to two sites in the λ DNA known as the operators¹. Repressor bound to the operator prevents the RNA polymerase from transcribing the two early operons of λ². This simple model is complicated by the fact that, unlike the *lac* operator³, the λ operator is a large and complex structure containing several repressor binding sites⁴. At low concentrations, repressor binds preferentially to a primary site which is about 30 base pairs long and is located at the operator end nearest the genes of the operon. At higher repressor concentrations, the remaining sites are progressively occupied, covering a total region approximately 110 base pairs in length. The two operators of λ, O_L and O_R, have a similar structure but differ somewhat in their affinity for repressor: O_L is reported to bind repressor about five times more tightly than O_R (ref. 4).

Maniatis *et al.*⁵ have reported a sequence for the primary repressor binding site in the O_L operator. They made use of the fact that O_L (as well as O_R) contains a site cleaved by the *Hind* II restriction endonuclease⁶. Treatment of O_L or O_R with this enzyme cuts off from the operator a fragment of 30 base pairs containing the primary repressor binding site (Fig. 1). Maniatis *et al.* were able to sequence this fragment utilising the cleavage product containing the remainder of the operator as a primer for repair synthesis by DNA polymerase.

Working with O_R, I have obtained a sequence of 80 nucleotides using RNA polymerase to transcribe operator fragments obtained by protecting the operator with repressor against nuclease digestion. This paper is a preliminary report and discussion of the possible implications of this sequence which starts in the middle of O_R and extends to the end of the operator including the primary repressor binding site.

O_R operator fragments were prepared in microgram amounts⁷

by digesting with pancreatic DNase the complex formed by excess λ repressor and several milligrams of sonicated DNA extracted from λdbio M30-7, a phage containing only the right-hand operator. The product, collected on nitrocellulose filters, extracted and purified by gel electrophoresis, was primarily a fragment about 110 base pairs in length as determined by gel electrophoresis. I used these fragments as a template for transcription by *Escherichia coli* RNA polymerase. Whether native or denatured, both strands were transcribed yielding a heterogeneous product ranging from very short chains to chains nearly as long as the template. This material, digested with RNase T₁, gave fingerprints containing a bewildering number of spots. Analysis of these nucleotides revealed, however, that many of them were related and resulted from the heterogeneous starts and stops made by the RNA polymerase. Making use of nearest neighbour information¹⁸ and analysis with pancreatic RNase and RNase U₂, I was able to obtain the sequence of the T₁ oligonucleotides but the heterogeneity of the product prevented further progress in establishing a total sequence.

Specific initiation

It has been shown by several workers⁸⁻¹⁰ that at sufficiently low nucleotide triphosphate concentrations, the RNA polymerase can no longer initiate transcription but can still elongate

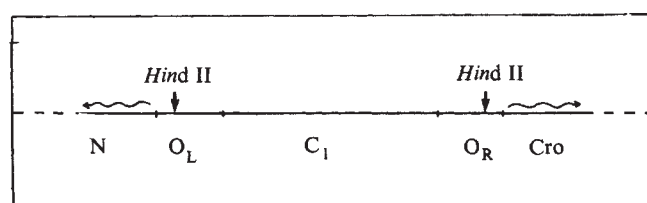


Fig. 1 Schematic map of the control region of phage λ. The primary repressor binding sites in O_L and O_R are located between the R.*Hind* II cleavage sites (↓) and the initiation of transcription (~~~~~→).

Fig. 3 Summary of the sequences obtained by primed transcription. The figure shows the sequence of the principal primed transcription products discussed in the text. The underlined nucleotides indicate the priming sequence. The nucleotides are numbered starting from the most frequently observed terminus of the operator fragment. The next nucleotide, numbered -1 is the site of transcription initiation *in vivo* (see the following article).

Table 1 ApApA primed transcription of O_R fragments

Band		Pancreatic RNase		RNase T_1	
		AAAU(C), C(U), U(A), AU(C), AC(C), C(G), C(A)		AAAUCUAUCACCG(C)	
1					
2	Same as band 1 +	GC(A), AAGGGAU(A), AAAU(A)	Same as band 1 +	CAAG(G), G(G), G(A)	
3	Same as band 2 +	AAC(A), GU(G)	Same as band 2 +	AUAAAUAUCUAACACCG(U), UG(C)	
4	Same as band 3 +	GU(U), U(G), GAC(U), GC(G)	Same as band 3 +	CG(U), UUG(A), UG(U)	
5	Same as band 4 +	U(U), U(G), GGC(G), GGU(G), U(C), AU(U)	Same as band 4 +	ACUAUUUUACCUCUG(G), G(C), UG(A), G(U), CG(G)	
6	Same as band 5 +	GAU(A), AAU(G), GGU(U)	Same as band 5 +	AUAAUG(G), UUG(C)	

sequence: 5'AAAUCUAUCACCGCAAGGGAUAAAUAUCUAACACCGUGCGUGUGACUAUUUUACCUCUGGCGGUGAUAAU-GGUUG(C)3'

Order of appearance of oligonucleotides in gel bands. Bands were cut out from the gels using the autoradiographs as a guide. The bands were extracted by soaking overnight in 1 ml 0.5 M NH_4OAc , 0.05% SDS and 30 μg tRNA. The extract was precipitated with 3 volumes of ethanol and the precipitate was digested with RNase T_1 or pancreatic RNase. Fingerprints were obtained by electrophoresis on cellulose acetate strips in the first dimension followed by homochromatography on DEAE-cellulose thin-layer plates¹⁹. The spots from the fingerprints were then analysed with RNase T_1 , pancreatic RNase, RNase U_2 or alkaline hydrolysis as required. The table lists only the new oligonucleotides appearing in gel bands of increasing size, but omits the 3'OH terminals. Each band also contains the nucleotides of the preceding band. The underlined nucleotides indicate the primer. The nucleotides in parentheses were deduced from experiments in which only one of the four nucleotide triphosphates was labelled. The labelled phosphate is transferred to the 3'OH end of the oligonucleotide which precedes it.

rebinding full size operator fragments with limiting amounts of repressor and redigesting the complex with pancreatic DNase. The resulting fragments were much shorter, ranging from about 30 to about 60 base pairs. They should still include the primary binding site but should have lost some of the weaker secondary sites. Transcription of these fragments, primed with ApApA, was initiated with the AAA at position 56 in the sequence shown in Fig. 3, indicating that it proceeds in the direction of the primary repressor binding site.

An alternative way to establish the orientation of the sequence is to localise it with respect to the *R.Hind* II cleavage site. I treated full size operator fragments with *Hind* II endonuclease, and separated the two products by gel electrophoresis. The larger fragment, approximately 80 base pairs long, transcribed with ApApA primer, yielded a sequence which is identical to that analysed in Table 1 but terminating with UpU_{OH} after 45 nucleotides (Figs 2b and 4). Transcription of the shorter fragment, approximately 30 base pairs long, with the same primer gave a mixture of products (Figs 2c and 4). Surprisingly, the major products are the result of incorrect priming: starting with AAAUG rather than with AAUG and with AAAC rather than with AAC. Priming at the AAAA site occurs less frequently and primarily starts with the first A rather than the second, yielding AAAAUAGU(C_{OH}).

I conclude from these results that the sequence obtained begins in the distal one third of the operator and proceeds to the end proximal to the operon and including the primary repressor binding site. Further confirmation of this sequence was obtained by sequencing the RNA polymerase binding site associated with O_R as reported in the accompanying article¹¹.

Sequence features

The position of the *R.Hind* II cleavage site acts as a point of reference for comparison with the sequence of the primary site of O_L (Fig. 5). In O_R , the *R.Hind* II cleavage site has the sequence 5' GTTGAC 3'. This together with the results of others shows that the *R.Hind* II recognition sequence GTTpyuAC¹² includes all four combinations of py and pu and not only the complementary pairs. There are therefore three sequences recognised by this enzyme, since GTTGAC and GTCAAC are complementary and represent the same sequence. Another reference point is the transcription initiation site which in O_R is located at the nucleotide immediately following the C at position 1 in the sequence¹¹. In O_R , as in O_L , the transcription initiation is located 33 nucleotides from the *R.Hind* II cleavage site. With these reference points, the O_L primary site sequence can be compared base for base with the O_R sequence.

The results of such a comparison are at present difficult to interpret. There is a high degree of homology between O_R and O_L and many sequences occur repeatedly in O_R . Such preservation or repetition of sequence features does not necessarily signify involvement in repressor recognition, however. It might be in part the result of recognition of other proteins (RNA polymerase, *tof* gene product) which interact with the same DNA region^{14,15}. Or it might simply be due to the evolutionary process which gave rise to the multiple operator and preserved irrelevant sequence features as well as important ones. In what follows I present one of several possible schemes for interpreting the O_R sequence in terms of repressor binding sites.

By comparing the O_R sequence with that of the O_L primary

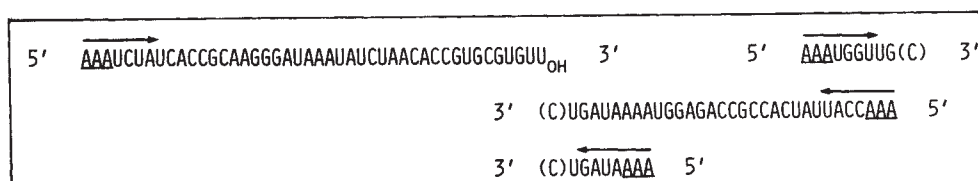


Fig. 4 Sequences obtained from the two O_R fragments produced by *R.Hind* II cleavage. The sequence on the left represents the largest product of transcription of the larger *R.Hind* II fragment. The sequences on the right, transcribed in both directions, are obtained from the smaller *R.Hind* II fragment. Two of these sequences are due to incorrect priming of ApApA at sites containing only two As.

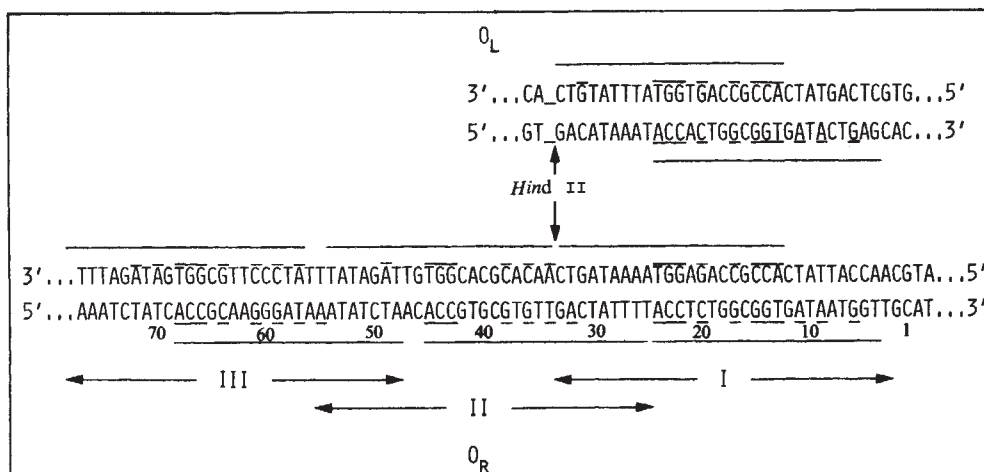


Fig. 5 Comparison of O_L and O_R sequences. Sequences in O_R comparable to the O_L sequence⁵, are marked I, II and III. These partially overlapping regions are proposed as binding sites for repressor tetramers. The location of the dimers is represented by solid lines which are not intended to imply that each dimer recognises only one strand. The underlined nucleotides indicate sequence elements most nearly common to the eight dimer binding sites.

site, it is possible to single out three partially overlapping regions which present substantial sequence homology with each other and with O_L . These regions, labelled I, II and III in Fig. 5, are 30–35 base pairs long and each would correspond to a complete recognition site for a repressor oligomer, most likely a tetramer, given the size of the region involved and the structure of the repressor oligomer seen in the electron microscope (Brack and Pirrotta, unpublished). Region I, which has the highest degree of homology with O_L , is of course the primary binding site of O_R .

It has been pointed out that many recognition sequences in DNA contain a high degree of symmetry. The *lac* operator sequence contains an axis of partial twofold symmetry in which 16 out of 21 base pairs participate³. In the λ O_L sequence, Maniatis *et al.* have detected three such axes. In the three O_R regions which I propose, this kind of symmetry is also abundantly present. Three axes of twofold symmetry can be distinguished in region I, centred at positions 17, 18 and between 18 and 19. Similar axes occur in regions II and III at corresponding positions in their sequence. It is difficult to interpret these symmetries in terms of repressor recognition because the nucleotides involved are not always the same from one sequence to another. While this kind of symmetry would permit the formation of self-complementary loops as proposed by Gierer¹⁷, experiments with superhelical DNA^{4,18} have recently shown that such structures are not involved in repressor binding. It seems more likely instead that the symmetry of the recognition sequence simply reflects the symmetry of the protein interacting with it. Both the *lac* and the λ repressors are oligomers of identical subunits and may be reasonably expected to possess an axis of twofold symmetry. A possible binding scheme for λ repressor subunits, which exploits one set of symmetry axes and can account for the results of protection experiments, is the following.

We can image two repressor dimers binding to two regions about 21 nucleotides long and overlapping in the middle, nucleotides 4–24 and 13–33 respectively. These two regions are in fact related by a twofold axis of symmetry located between nucleotides 18 and 19, which would permit the two dimers to recognise similar sequences on opposite strands. In the O_L sequence, this interpretation would account for the second of the axes of symmetry detected by Maniatis *et al.*⁵. An additional dimer binding to O_R would occupy the region between nucleotides 25 and 45: the first half of region II. This arrangement would protect against nuclease digestion a region of approximately 45 base pairs corresponding to the S_1+S_2 fragment reported by Maniatis and Ptashne⁴. Region II is the one with the lowest degree of homology with the O_L

sequence. We may expect that repressor binding to this site is weakest and stabilised only by interaction with the repressor bound at the primary site. Binding of additional repressor dimers would then fill the remaining sites, progressively increasing the size of the protected region in steps of approximately 15 base pairs. At each step, the partial overlap of the sites would permit interaction of a dimer with the preceding one.

This model yields eight sequences, two from O_L and six from O_R , which are now known and which would constitute binding sites for repressor dimers. While differing in many details, these sequences have certain features, underlined in Fig. 5, which remain remarkably constant and are nearly common to all eight proposed binding sites. According to this model, these nucleotides are therefore the most likely candidates for repressor recognition elements and for the sites of operator constitutive mutations. The only such mutation so far identified is in fact located at position 24 in O_L (ref. 5).

It will be of great interest to see whether the sequence of the remaining sites of O_R and O_L conforms to this pattern and whether other operator mutations are in fact located as predicted by the model.

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Sequence of the P_R promoter of phage λ

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The RNA polymerase binding site from the λ P_R promoter was isolated and sequenced. This DNA fragment contains the transcription initiation site and shares 25 nucleotides with O_R . The sequence preceding the initiation site suggests that the promoter recognition site is not identical with the tight binding and initiation site.

THE promoter has been loosely defined as the site on the DNA where the RNA polymerase recognises some signal which allows it to bind tightly and initiate transcription. There are several such physiologically significant sites in the DNA of phage λ , two of which are the early promoters controlled by the repressor and governing transcription of the early genes, to the right and to the left of the two operators. Genetically these promoters are located very near the operators. Some promoter mutations have been mapped between known operator mutations, suggesting that the two sites may overlap¹. Allet and Solem² and Maurer *et al.*³ have shown that certain promoter mutations alter a recognition site for the *Hind* II restriction endonuclease, which is located about 30 base pairs inside the operator. This finding implies that the operator and promoter overlap at least partially.

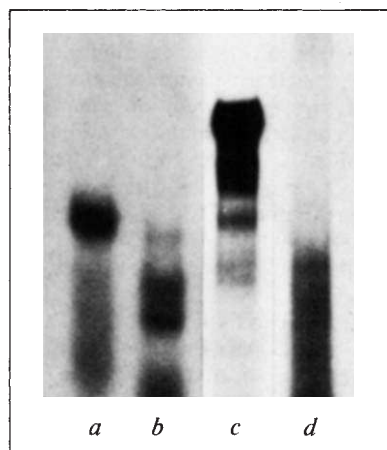


Fig. 1 Gel electrophoresis of RNA polymerase protected fragments. Operator-containing fragments were preselected from 500 μ g sonicated λ dbio M30-7 DNA labelled with 32 P, by binding with λ repressor and filtering through a membrane filter¹¹. 5 μ g RNA polymerase holoenzyme were added to the preselected fragments in buffer containing 0.05 M Tris, pH 7.9, 0.1 M KCl, 0.01 M $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithioerythritol and 5% glycerol. After 10 min at 37 °C, pancreatic DNase was added to a concentration of 100–200 μ g ml⁻¹ and incubation continued for another 10 min. If the KCl concentration was higher than 0.1 M, the DNase was not sufficiently active unless the reaction mixture was diluted. The digestion was stopped by addition of excess EDTA immediately followed by a phenol extraction and precipitation with 2 volumes of ethanol. The precipitate was resuspended and applied to a 10.4% acrylamide–0.7% agarose gel made up in 0.09 M Tris-Borate, pH 8.2, and 2.5 mM EDTA. After electrophoresis for 2 h at 25 mA, the gel was autoradiographed. *a*, RNA polymerase protected fragments; *b*, heat denatured protected fragments; *c*, operator fragments of mixed sizes prepared as previously described¹¹; *d*, product of digestion of operator fragments in the presence of excess RNA polymerase. The polymerase does not protect any part of the operator in these conditions.

In the *lac* operon, the polymerase initiates transcription at the distal end of the operator and the mRNA produced contains the operator sequence^{4,5}, whereas in λ this is not the case. It has been shown, at least for the left-hand operon, that transcription initiates outside the immunity region⁶ and in fact immediately outside the primary repressor binding site⁷.

One approach to the study of the promoter has been to isolate the region of DNA with which the polymerase forms a stable complex. At temperatures above 20 °C, RNA polymerase binds extremely tightly to promoter sites, forming the so-called "open complexes" from which it is able to initiate transcription⁸. This tight binding has been exploited to isolate DNA fragments protected by RNA polymerase against nuclease digestion^{9,10}.

We have used the same approach to isolate a polymerase protected fragment from the vicinity of the λ O_R operator. Here we report the isolation and properties of this fragment and its sequence, obtained by primed transcription of the isolated fragment.

Isolation of polymerase protected fragments

To minimise nonspecific polymerase binding we used as starting material DNA fragments from the immediate vicinity of O_R . These fragments were obtained by sonicating the DNA of λ dbio M30-7, a phage containing only the right-hand operator, and selecting the operator-containing pieces by binding them with repressor and trapping them on a nitrocellulose membrane filter. We then added RNA polymerase holoenzyme in limiting

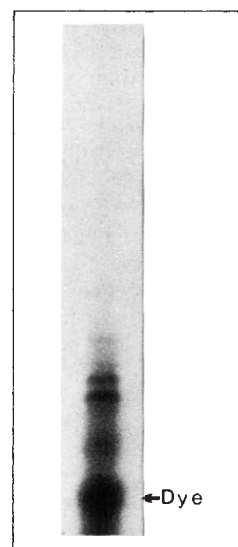


Fig. 2 Gel electrophoresis of transcription initiated on RNA polymerase–DNA complexes. Complexes of RNA polymerase and preselected DNA fragments were prepared and digested with pancreatic DNase as in Fig. 1. After 10 min of digestion at 37 °C, 3 unlabelled NTPs were added to the same mixture at a concentration of 0.2 mM and the fourth, α - 32 P labelled at 100 mCi μ mol⁻¹, and a concentration of 5 μ M. After incubation for 0.5–2 min, the reaction was stopped by adding excess EDTA and precipitating with 3 volumes ethanol. The precipitate, resuspended in 0.009 M Tris-Borate–7 M urea, was heated in boiling water for 2 min and applied to a 12% acrylamide slab gel containing 7 M urea and electrophoresed as described in ref. 14.

Table 1 Transcription initiation sequence

Pancreatic RNase	RNase T ₁
pppAU (G), GU (A), AC (U), U (A), AAGGAGGU (U), U (G) pppAUGUACUAAGGAGGUUG	pppAUG (U), UACUAAG (G), G (A), AG (G), G (U), UUG

Bands were cut out from the slab gel and extracted as described previously¹⁴. The band contents were digested with pancreatic RNase and RNase T₁ and the products separated by electrophoresis on cellulose acetate pH 3.6 in the first dimension, followed by chromatography on PEI cellulose thin layers with 2.5 M pyridine formate pH 3.5 and 7 M urea. The sequence of the oligonucleotides were determined by analysis with pancreatic RNase, RNase T₁, and by nearest-neighbour information (represented in parentheses).

amounts and digested the resulting complex with pancreatic DNase. The digestion was stopped by a phenol extraction, and the DNA fragments protected against digestion, which we will refer to as polymerase-protected or P-fragments, were then precipitated with ethanol and analysed by gel electrophoresis. The P-fragments migrated as double stranded DNA about 40–45 base pairs long (Fig. 1). Heat denatured P-fragments migrate faster and are clearly distinguishable. While isolated λ operator fragments can be shown to rebind well to repressor^{11,12}, the P-fragments do not rebind to RNA polymerase. Nor could we demonstrate binding of repressor to the P-fragment. Furthermore, polymerase could not protect any part of the isolated operator fragments against DNase digestion. Treatment of the P-fragments with the *Hind* II endonuclease produced no detectable change in size. While these are all negative results, taken together they suggest that if there is overlap between the repressor binding site and the polymerase binding site, it is only partial and does not extend as far as the R. *Hind* II cleavage site.

Transcription initiation site

The RNA polymerase protected fragment contains the initiation site for transcription. We treated the polymerase–DNA complex as before with pancreatic DNase but we then added the nucleotide triphosphates to initiate transcription in the same reaction mixture. As the polymerase starts transcribing, it exposes the protected DNA to digestion by the DNase. Each polymerase complex can therefore initiate only once and from a unique site in the protected fragment. On gel electrophoresis the transcription product consists of two bands differing in length only by a few nucleotides. The largest product is 17–18 nucleotides long (Fig. 2). The fingerprints show that the two bands represent a unique sequence initiating with pppAUG (Table 1) and corresponding to the sequence of the normal transcription initiation from P_R observed both *in vivo* and *in vitro*⁶.

We conclude that, as was previously observed by Schaller *et al.*¹³ with fd RF DNA, the site at which the RNA polymerase forms the tight complex is also the site from which it initiates transcription and that the active site of the polymerase is located at about one-third of the total distance covered by the enzyme bound to the DNA.

Primed transcription

To sequence the remaining part of the polymerase binding site we used again the RNA polymerase but this time forced transcription of the isolated protected fragments using di- or trinucleotide primers and a denatured template. Since the initiation sequence in Table 1 contains two GG sites, we used CpC as a primer, hoping to obtain transcription in the opposite direction. Gel electrophoresis of the transcription product separated several bands which, when analysed with RNase T₁, proved to represent a single growing chain (Fig. 3 and Table 2). The last oligonucleotides to appear corresponded to those found in the transcription initiation sequence in Table 1, showing that, contrary to our expectation, the CpC was priming in the same direction but starting near the other end of the protected fragment. The order of appearance of the RNase T₁ and pancreatic RNase oligonucleotides, together with nearest-neighbour information, sufficed to establish a sequence of 40 nucleotides, starting with the CpC primer and ending with the transcription initiation sequence. Since the entire protected fragment is 40–45 base pairs in length, no more than five nucleotides could still be missing. To determine these and to confirm the sequence, we attempted again to prime transcription in the opposite direction, using this time ApUpG as a primer. This primer in fact gave transcription in both directions, separable by gel electrophoresis and made it possible for us to add a maximum of three additional nucleotides to the sequence (Table 3). These were not always present and probably represent the ragged ends left by the pancreatic DNase treatment used to prepare the protected fragments.

The promoter–operator region

The sequence we have obtained for the RNA polymerase binding site shares about 25 nucleotide pairs with the sequence of the primary repressor binding site of the λ O_R operator¹⁴. Only the transcription initiation sequence is unique to the polymerase site (Fig. 4). The overlap between operator and polymerase site is therefore only partial and does not extend to the region recognised by the *Hind* II endonuclease. How can we reconcile these findings with the observation that promoter mutations are located in the R. *Hind* II site and that polymerase bound to λ DNA prevents the *Hind* II enzyme from cleaving the operators^{3,9}? We note that the latter effect was observed at a molar ratio of 30 polymerase per DNA, and could be easily explained by steric hindrance preventing the approach of the *Hind* II enzyme or by multiple binding of polymerase in the promoter region¹⁶. The former observation however, implies that the polymerase recognises a region including the R. *Hind* II sequence. It is unlikely that it could do so while bound at the initiation site since the polymerase molecule itself, as seen in the electron microscope, is not longer than the 40 base pair region it protects (Brack, personal communication). One possible explanation is that the polymerase requires additional sequence elements for recognition and 'entry' into the DNA. This would explain why the isolated protected fragments do not have

Table 2 Sequence of CpC primed transcription

Band	Pancreatic RNase	RNase T ₁
1	C (U), U (C), U (G)	<u>CC</u> UCUG (G)
2	GGC (G), GGU (G)	same as band 1 + G (C), CG (G), G (U)
3	GAU (A)	same as band 2 + UG (A)
4	AAU (G), GGU (U), U (G), GC (A)	same as band 3 + AUAAUG (G), G (U), UUG (C)
5	AU (G), GU (A), AC (U), U (A) AAGGAGGU (U), U (U), U (G)	same as band 4 + CAUG (U), UACUAAG (G) G (A), AG (G), G (U), UUG
sequence 5' <u>CC</u> UCUGGCGUGAUAAUGGUUGCAUGUACUAAGGAGGUUG . . . 3'		

Bands were cut out from the gel in Fig. 3 and fingerprinted as described in Table 1. Only the new oligonucleotides appearing in each band are listed. Each band contains also the oligonucleotides of the preceding band. The underlined nucleotides indicate the primer sequence.

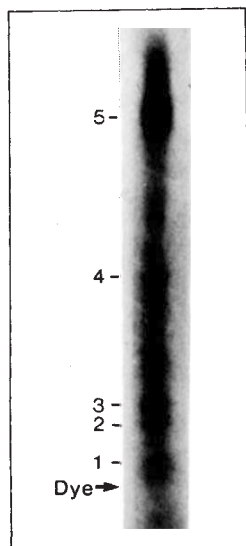


Fig. 3 Gel electrophoresis of primed transcription. RNA polymerase protected fragments were prepared as described in Fig. 1 and used as templates for transcription primed with CpC. The details of the transcription reaction and urea gel electrophoresis were as described in ref. 14. The oligonucleotide composition of the numbered bands is shown in Table 2.

appreciable affinity for RNA polymerase. We propose then that recognition, entry, tight binding and finally initiation are different elements of the polymerase-promoter interaction which need not occur at exactly the same site. The last two events clearly take place at the site whose sequence we report here. The site for the first and second events must be looked for further inside the operator sequence. In this region, however, it is difficult to discern which structural features of the sequence result from the repressor binding function and which result from the polymerase recognition. Nevertheless, by comparison with the sequence of other promoter regions we can discern two common features: First the presence of one or more AAAT sequences, and second, regions of very high GC content flanking a region of very high AT content.

Similar features have been noted in the *lac* promoter region (5) and in that of the *tRNA^{Phe}* gene¹⁶ and it has been suggested that they may be involved in the recognition and entry process. Furthermore, if the λ operator-promoter sequence is aligned with that of the *lac* promoter-operator region using the transcription initiation site as a reference, the GC and AT-rich regions are almost exactly in register and 22 base pairs out of 48 are identical. We suppose that a loose complex is formed by the

polymerase at the recognition or entry site. Transition of this loose complex to an 'open complex' might result from the opening of some 6 base pairs, in the AT-rich region. The tight binding, however, occurs only when the polymerase shifts from the entry site to the binding or initiation site. This shift must occur fairly rapidly after 'opening' of the promoter and does not require the presence of the nucleotide triphosphates. We anticipate that, in the appropriate conditions, it should be possible to block the polymerase at the recognition or entry site and identify the sequence involved. Dickson *et al.*⁹ have proposed a similar model for the *lac* promoter. They suggested that the role of the GC-rich regions might be to act as a "transducer"; that is to transmit the helix destabilisation produced by the catabolite gene activator protein (CAP) bound to the region immediately adjacent to the GC-rich region and so facilitate entry by the polymerase. The fact that the λ P_R promoter has the same GC-AT-GC structure even though it is not regulated by the CAP protein suggests that this structure may be a more general feature of polymerase recognition sequences. We note that in the P_R sequence the GC region contains only 73% GC rather than 83% as in the *lac* region. This difference might be enough to make polymerase entry into the λ P_R promoter possible without additional facilitation. It is possible that the transition temperature to an 'open' RNA polymerase-promoter complex is related to the GC content of the regions flanking at the AT-rich region.

Finally, we would like to point out an additional feature of the promoter-operator region, which may be significant. The sequence contains three blocks of high GC content alternating with two blocks of high AT content. Furthermore, two axes of relatively high partial symmetry are located at, or adjacent to, nucleotide 41 (Fig. 4). These two observations suggest the possibility that there may be two polymerase recognition or entry sites in this region, both composed of two GC-rich regions surrounding an AT-rich region. The two sites would share one GC-rich block, and, because of the rotational symmetry, would be oppositely oriented. We know in fact, that there exists a promoter for leftward transcription in this region. The P_{rm} or repression maintenance promoter is a weak promoter responsible for transcription of the λ C_1 or repressor gene in a lysogenic cell¹⁷. A mutation in this promoter has been localised in the region of nucleotides 48-54 in the O_R sequence (Hedgpeth and Smith, personal communication). Furthermore, *in vivo*, functioning of this promoter requires the presence of active repressor^{17,18}. If the region between nucleotides 35 and 71 is in fact the recognition-entry site of this promoter, we can easily explain this requirement. P_R is a strong promoter: it has a high affinity for polymerase. Polymerase binding at the P_R recognition site is incompatible with binding at the P_{rm} site. Hence, in the absence of repressor, P_R transcription predominates. In the presence of repressor, P_R is pre-empted by repressor bound at the primary site of O_R , occupying the region 1-33 in the sequence.

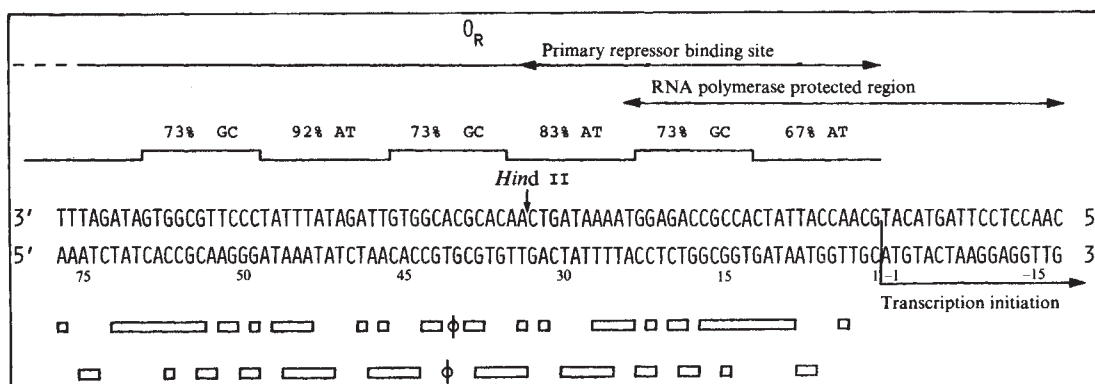


Fig. 4 Promoter-operator sequence from the O_R region. The RNA sequences are written as DNA. The regions of high GC and high AT content are marked above the sequence. The bars below the sequence indicate complementary sequences located symmetrically with respect to two possible axes of symmetry at or adjacent to nucleotide 41.

Table 3 Sequence of ApUpG primed transcription

Band	Pancreatic RNase	RNase T ₁
1	GU (A), AC (U), U (A), AAGGAGGU (U)	AUG (U), UACUAAG (G), G (A), AG (G), G (U)
2	GC (A), AAC (C), C (A), AU (U), U (A), AU (C), AC (C), C (G), GC	AUG (C), CAACCAUUAUCACCG (C)
3	Same as in band 2 + AGAGGU (A)	Same as in band 2 + CCAG (A), AG (G), G (U), UA _{OH}
Sequence 3' ^{HO} AUGGAGACCGCCACUAUUACCAACGUA 5'		5' AUGUACUAAGGAGGU (U) 3'

The sequence of the largest T₁ oligonucleotide was not determined in detail but was deduced by pancreatic RNase analysis and from the sequence of the complementary strand.

This leaves P_{rm} available to polymerase for transcription of the C₁ gene. If excess repressor is present, the additional repressor binding sites will begin to be occupied, pre-empting also P_{rm} and therefore regulating by negative feedback the transcription of the repressor gene itself. A prediction generated by this model is that if the P_R site is blocked by suitable amounts of repressor, or better, by cleavage with R. *Hind* II it should be possible to detect leftward transcription *in vitro* of the O_R-C₁ region starting at the P_{rm} site.

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Enhancement of the electrical excitability of neuroblastoma cells by valinomycin

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Mouse neuroblastoma cells in stationary phase of growth display partially developed electrical properties. Addition of the K⁺ selective carrier valinomycin to these cells causes rapid enhancement of electrical excitability. We suggest that the appearance of molecules with properties similar to valinomycin is essential for the full expression of electrical excitability in differentiating neuroblastoma.

MACROCYCLIC antibiotics and related compounds which can selectively translocate ions in artificial and biological membranes, have been used to explore the molecular basis of cellular ion permeation¹⁻³. The natural occurrence of ionophores in biological membranes has been reported^{4,5} and supports the speculation that cyclic molecules, especially those with valinomycin-like properties, may provide the natural basis for resting membrane permeability in some tissues^{3,6}. The application of compounds such as valinomycin to electrically excitable membranes, however, either did not produce detectable permeability changes⁷⁻⁹, or gave rise to an impairment of electrical function^{7,10,11}. As the membranes already studied possessed highly specific permeability properties, we wished to investigate an excitable system in which resting and active selectivity patterns are not fully established. Mouse C-1300 neuroblastoma, from which many clonal lines have been isolated^{12,13}, is particularly suitable. Cells from several lines can generate well developed action potentials after long term treatment with certain agents such as aminopterin or

dibutyryl cyclic AMP (db-cyclic AMP)¹⁴⁻¹⁶, but as we show here, if these cells are maintained in the stationary phase of growth, excitability is only partially expressed. The transition from partial to full excitability, a process normally taking 1-3 weeks, includes a rise in resting potential, membrane resistivity and action potential currents. Here we show that a comparable change in electrical properties can be rapidly induced by the addition to the culture medium of low concentration of valinomycin.

Properties of stationary phase cells

Electrophysiological experiments were performed on clone N1E-115 cells in stationary phase of growth using previously described techniques for intracellular microelectrode recording¹⁵. Because the confluent cell population is somewhat heterogeneous, only large cells of diameter of 70-100 μm with a marked degree of process formation were studied (see Fig. 3a). As summarised in Table 1, transmembrane resting potentials and input membrane resistances were relatively low for most of these cells. When equal depolarising and hyperpolarising current pulses were applied at the resting potential, most cells showed delayed rectification, that is, smaller resistance with depolarising than with hyperpolarising pulses¹⁷. Figure 1a shows that with sufficiently large depolarising pulses, the elicited voltage reached a maximum, then gradually declined to a plateau 2-8 mV below the peak. As shown in Fig. 2 increments in the intensity of the depolarising pulse gave only slight

Table 1 Effects of valinomycin on electrical properties of stationary phase N1E-115 neuroblastoma cells

	Membrane potential (mV)*	Membrane resistance (M Ω)	Time constant (ms)	Max. dV/dt rise (V s ⁻¹)	Max. dV/dt fall (V s ⁻¹)	Potential at spike peak (mV)	Amplitude of fall phase (mV)	<i>n</i>
Control	16.4 $\pm$ 1.4	5.5 $\pm$ 0.5	7.8 $\pm$ 1.0	21.1 $\pm$ 2.9	2.3 $\pm$ 0.4	7.6 $\pm$ 1.7	20 $\pm$ 1.0	37
Valinomycin treated (10 ⁻⁶ –10 ⁻⁸ M)	34.3 $\pm$ 3.8	12.5 $\pm$ 1.8	15.3 $\pm$ 2.1	53.6 $\pm$ 6.8	28.5 $\pm$ 3.2	24.7 $\pm$ 2.0	52.5 $\pm$ 2.0	16

Cultures were grown to confluency in Dulbecco–Vogt modified Eagle's MEM (DMEM) (GIBCO), supplemented with 10% foetal calf serum (FCS) (GIBCO) in Falcon plastic flasks. For electrophysiological studies, cells that were maintained in confluency for at least 2–4 weeks with daily changes of medium were washed twice with Puck's saline D1 and then treated with D1 containing 0.05% trypsin for 5 min. After resuspension in fresh DMEM + FCS, 5×10^5 cells were seeded per 60 mm Falcon tissue culture dish (2.4×10^4 cells/cm²). The replated cells passed through a lag period of 4–5 d before cell division recommenced; this continued for 4–5 d until cells reached a high density confluent state (3×10^6 cells per 60 mm dish, or 1.9×10^5 cells/cm²). Electrical studies were made on both lag and confluent cells, their electrical properties were found to be similar. In parallel, cells from the same flasks were plated at a similar density for determination of intracellular ionic concentration by atomic absorption spectrophotometry (in preparation). Stock solutions of valinomycin (Calbiochem) at 10^{-3} M were prepared in 100% ethanol, and diluted on the day of the experiment at least 1,000-fold to the concentration used in the experimental media. The ethanol concentration which never exceeded 0.2% was found to have no effect on the electrical properties of neuroblastoma cells. In the present set of experiments the membrane was exposed to a concentration of 10^{-8} M valinomycin after recordings were obtained from cells in normal medium. In one of two valinomycin batches used, the initial concentration of 10^{-8} M was found to be effective. But in the second batch, the effective concentration was 10^{-6} M. Similar differences in valinomycin activity between batches have been seen previously and result most probably from deterioration^{23, 20, 21}.

Intracellular microelectrode recordings were made in the culture dish which was held in a special chamber on the stage of an inverted phase-contrast microscope with control of temperature between 36 and 38 °C. The pH was maintained between 7.2 and 7.4 by passing a constant stream of warmed 5% CO₂ in air over the dish. The cultures were bathed in the growth medium which contained the following salt concentrations in mmol l⁻¹: NaCl-109.5; KCl-5.5; CaCl₂-1.8; MgSO₄-0.8; NaHCO₃-14.4; NaH₂PO₄-0.9. 3M-KCl micropipettes with resistance ranging from 10–20 M Ω were used. They were arranged in the bridge circuit of a Bioelectric P system to allow stimulation of the cells through the recording pipette. The transmembrane voltage and stimulating currents as well as the electronically derived time derivative of transmembrane voltage were displayed on an oscilloscope and photographed. When a stable resting membrane potential had been reached, input membrane resistance and an estimate of membrane time constant were determined using comparable hyperpolarising current pulses. Determinations of active membrane properties were made on cells adjusted to a standard membrane potential level of -95 ± 5 mV. This gives a nearly maximal rate of rise and eliminates variation as a result of differences in initial resting potential. Ionic currents flowing during an evoked action potential were quantitatively estimated from the maximum value of the time derivative of the transmembrane potential (maximum dV/dt)¹⁵. Responses were also measured in terms of the differences between the maximum membrane depolarisation evoked by the stimulating pulse and the adjusted steady membrane potential (potential at spike peak). Positive values of this measure indicate action potential overshooting zero potential.

*Values in all columns are given as mean $\pm$ s.e.

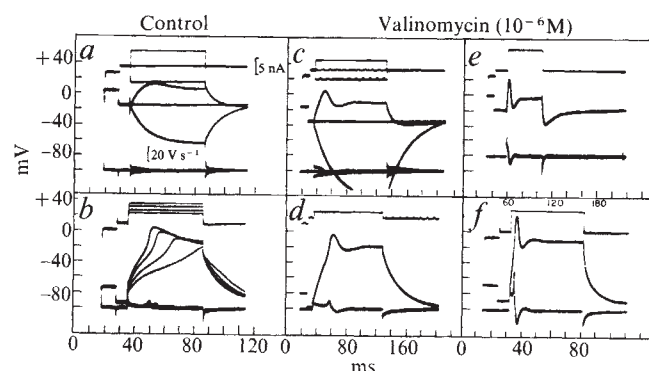


Fig. 1 The effects of valinomycin on the electrical activity of stationary-phase neuroblastoma N1E-115 cells. *a* and *b*, control records; *c* and *d*, *e* and *f*, examples of responses in the presence of valinomycin. All records are from the same dish; cells were replated 12 d before experiment. In this and subsequent figures the uppermost trace represents the injected current, inward current shown as downward deflection; the middle trace represents the transmembrane potential recorded in a bridge circuit; the lowest trace represents the time derivative of the transmembrane potential (dV/dt). Current and dV/dt calibration bars (in *a*) apply to all records. A 10 ms, 20 mV voltage calibration pulse is seen at the left of the middle traces. Records *a* and *c* represent the voltage responses to depolarising and hyperpolarising current pulses applied at the resting potential of each cell. Note that in the presence of valinomycin a smaller current intensity elicits a much larger hyperpolarising potential. In *e* an outward pulse was applied at a steady membrane potential adjusted to that of the control cell. Note the fast declining phase and hyperpolarising after potential. In *b*, *d* and *f* the steady membrane potential was adjusted to -100 mV to obtain maximal action potentials. Note that in the control (*b*), the slow time course of the falling phase is not affected by increments in the intensity of the applied current, whereas the rise rate is slightly increased; in *d* a stimulus at the threshold level was applied to show that after valinomycin treatment rise rates comparable with those in the control determine faster fall rates; *f*, a fast rising, short duration action potential followed by a slowly rising peak elicited by a larger stimulus in the presence of valinomycin.

changes in the steady potential level, whereas an almost linear current–voltage (I – V) relationship was obtained for inward currents. Few cells showed anomalous (inward-going) rectification (Fig. 3b)¹⁸, or ohmic behaviour throughout the explored range.

Active membrane properties were assessed by applying depolarising current pulses at a membrane potential preset to a standard level of -95 ± 5 mV. Under these conditions, all cells tested generated action potentials. As shown in Table 1 and Fig. 1b, the amplitude and rate of change of both rising and falling phases were generally low, and overshoot potentials could not always be recorded.

The electrical properties just described contrast strongly with the well developed properties obtained after long term cell selection with aminopterin or db-cyclic AMP^{15, 16}, suggesting that a major step which is required for full expression of electrical excitability does not take place in stationary phase cells. This step may involve a change in the permeability properties of the membrane. If this change includes an increase in K⁺ permeability (P_K), it seemed possible that valinomycin, a K⁺ selective carrier^{19–23}, could rapidly produce an enhancement of electrical excitability.

Effect of valinomycin on passive and active membrane properties

Table 1 compares electrical properties of cells before and after treatment with valinomycin. Steady state conditions were attained about 10 min after exposure to valinomycin and remained constant for experimental periods of at least 4 h. As shown, a concentration as low as 10^{-8} M produced a twofold increase in average resting potential accompanied by a similar increment in input membrane resistance and a dramatic enhancement of electrical excitability.

In terms of the constant-field equation^{24, 25} an increase in P_K which lowers the permeability ratios P_{Na}/P_K or P_{Cl}/P_K or both can explain the observed increase in resting potential provided

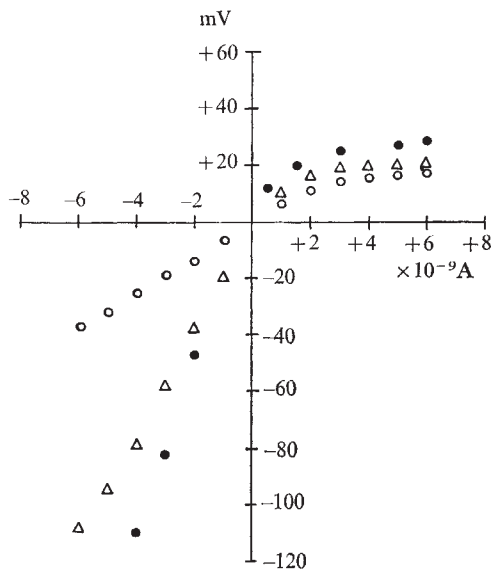


Fig. 2 Steady-state current-voltage (I - V) relationships in stationary-phase N1E-115 cells. $\circ$, Before; $\bullet$, after addition of valinomycin (10^{-8} M) to the culture medium. Cells were replated 3 d before the experiment. $\triangle$, I - V curve of a cell grown for 18 d in a culture medium containing aminopterin (10^{-7} M). The points represent the amplitude of the potential 150 ms after the start of the current pulse. Zero on the voltage axis corresponds to the resting potential of each cell which was -34 mV in all cases.

that the internal K^+ level remains constant. Preliminary measurements of intracellular K^+ concentration in replicate plates before and after 1 h incubation with 10^{-8} M valinomycin suggest that the antibiotic does not significantly alter the internal level of this ion, in both cases values of 155 ± 5 mM were obtained.

Using this value against an external K^+ concentration of 5.5 mM, an equilibrium potential for K^+ (E_K) of approximately -89 mV is obtained with the Nernst equation. This figure is substantially higher than that obtained experimentally for valinomycin-treated cells (mean value -34 mV) or that seen by us in aminopterin-selected cells (mean value -38 mV), and suggests that Na^+ and/or Cl^- ions continue to play a role in determining the resting potential.

If the hyperpolarisation of the membrane potential reflects an increase in P_K alone, then it should be paralleled by a rise in total resting membrane conductance, that is, by a fall in membrane resistance. Table 1 shows that valinomycin induces a twofold increase in the average input resistance. Thus, the steady-state I - V curve in the presence of valinomycin, resembling that obtained after aminopterin selection, exhibits a much steeper slope in the hyperpolarising direction (Fig. 2). Furthermore, although the voltage response evoked by a depolarising pulse was generally somewhat increased, as is illustrated in Fig. 3 all cells displayed delayed rectification. The unexpected increase in resistance indicates an overall diminution in the total number of passively conducting pathways. The most likely interpretation for this effect is that the fraction of membrane conductance contributed by Na^+ or Cl^- ions or both is lowered to an even greater extent than the increase in K^+ conductance.

The valinomycin induced changes in active membrane properties were of similar complexity. At membrane potentials positive to the threshold for spike initiation, when a depolarising stimulus elicited an early peak which declined to a steady-state level, valinomycin markedly increased the rate and amplitude of the decline (Fig. 1e). When spikes were initiated from the same hyperpolarised membrane potential as that of the control, the falling phase in particular exhibited a remarkable increase in both amplitude and maximum dV/dt (Table 1). This usually led to a considerable shortening in spike duration and to the appearance of an otherwise masked second, slowly rising peak (Fig. 1f). As the falling phase of the action potential is caused by an increased permeability to K^+ ions these results can be accounted for by an increased translocation of the valinomycin- K^+ complex across the excited membrane.

As shown in Table 1, valinomycin also had dramatic effects on the amplitude and rate of change of the depolarising phase. The current during this phase is carried by two systems: a tetrodotoxin (TTX)-sensitive, fast conducting Na^+ system, and a TTX-insensitive, slow conducting Ca^{2+} system¹⁵. The generally low maximum dV/dt values obtained with stationary cells indicate that the fast conducting Na^+ system is poorly developed at this stage. The significant increase in rise rates suggests therefore that valinomycin activates membrane elements involved in fast inward movement of Na^+ ions.

Estimation of the threshold potential for spike initiation from the inflection point between the slowly rising electrotonic potential and the upstroke of the action potential gave a value of about

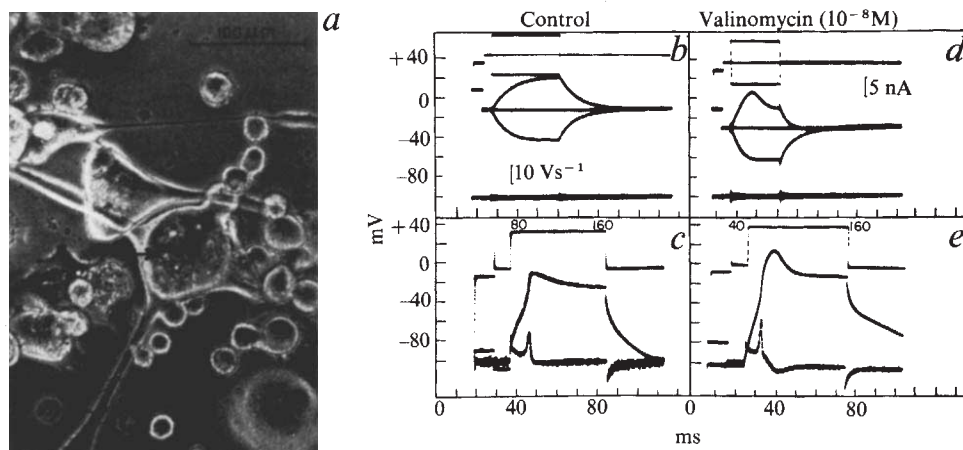


Fig. 3 Electrical activity from the same neuroblastoma cell before and after valinomycin treatment, *a*, Phase contrast photomicrograph of cells from a 'confluent' culture replated, 24 d before experiment. All records were obtained from the cell designated by an arrow in *a*. Voltage responses to depolarising and hyperpolarising current pulses applied at the resting potential of the cell are presented before (*b*), and after (*d*), addition of valinomycin (10^{-8} M) to the bath. Note the anomalous rectification before treatment and the delayed rectification in the presence of valinomycin. Action potentials are presented in records *c* and *e* before and after valinomycin treatment, respectively. In both cases the steady membrane potential was adjusted to about -100 mV to obtain maximal responses. Note that in the presence of valinomycin, maximum dV/dt and amplitude of both the rising and falling phases of the action potential were markedly increased. dV/dt calibration bar in *b* applies to *c*, *d* and *e*.

—40 mV both before and after treatment, so the rise in peak overshoot after valinomycin treatment represents an absolute increase in spike amplitude. Such an increase can be accounted for either by a change in electrochemical driving force for Na^+ ions, or by an increase in the voltage-dependent changes in Na^+ permeability during the upstroke of the action potential. Preliminary measurements of intracellular Na^+ concentration gave a value of 35 mM before treatment which declined to 27 mM after 1 h incubation with 10^{-8} M valinomycin. Calculating E_{Na} from the Nernst equation with these figures, against an external concentration of 124.8 mM, gives values of +34 and +41 mV respectively. Because such a small increase in E_{Na} cannot account for the large differences in peak action potential amplitudes before and after valinomycin treatment, it seems that a change in the rate at which membrane permeability to Na^+ increases during spike activity is involved.

Mode of action

Although the increase in resting potential and the enhanced repolarising electrogenesis can be easily rationalised on the basis of the known properties of valinomycin as a selective carrier of K^+ , the closing of passively conducting ionic pathways and the activation of membrane elements involved in the fast inward movement of Na^+ ions, underline the possibility that a structural rearrangement of the membrane takes place. This may include a modification of membrane structure by valinomycin unrelated to its ionophoric function. It is also possible that the ionic selectivity characteristics of the antibiotic could be modified by such determinants of membrane organisation as surface charges, dipoles and fluidity²⁶. Nevertheless, the striking similarity between the electrical properties of valinomycin-treated cells and those selected by long term incubation in aminopterin or db-cyclic AMP¹⁴⁻¹⁶ and the fact that changes in K^+ permeability seem to play a major role in many other developing cellular systems²⁷⁻³⁰, strongly suggest that this permeability change is responsible for the rearrangement of the neuroblastoma membrane. If so, this phenomenon could shed some light on the temporal relationship between surface membrane permeability changes and other events that take place during development^{31,32}. That such changes in neuroblastoma can be so rapidly triggered by valinomycin

leads us to propose that during the differentiation of these cells molecules with properties similar to those of valinomycin appear. These molecules are responsible for the induction of K^+ permeability, an event which seems to be crucial for the full expression of electrical excitability.

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letters to nature

Non-variable 13.5 mm flux in the strong millimetre component of Centaurus A

It has been suggested¹ that the peculiar galaxy and strong radio source Cen A has a strong and possibly variable component in the millimetre wavelength region.

Our observations do not support this claim. The evidence of variability was based on two measurements made at 3.4 mm and one at 9.5 mm at the 36-foot telescope of the National Radio Astronomy Observatory. Observations made at the position of the strong infrared source² were carefully calibrated against the sources of known intensity: Mars, Saturn and DR21. An important consideration in these observations was the relatively large correction for the atmospheric attenuation. At the NRAO site, Cen A has a maximum elevation of 15°. The atmospheric attenuation factor at the position of the source, $\exp(\tau)$, was about 1.5. The March 1974 measurements at 3.4 mm gave

results of 22.5 ± 2.0 Jy and 14.0 ± 1.5 Jy, and the result at 9.5 mm was 23.7 ± 2.0 Jy. If the errors cited are realistic estimates, the statistical significance of the detection of variability is very high.

We have made 18 measurements of Cen A at the position of the infrared source at a wavelength of 13.5 mm on nine days between April and December 1974. The observations were made with the 13.7-m radio telescope of the Itapetinga Radio Observatory³. The results of our observations of the flux density F_ν are given in Table 1 with the 1- σ statistical errors, s . We also present the observations of the reference source, Virgo A, which is assumed to have a flux density of 21.4 Jy at this wavelength⁴. Although the zenith attenuation at this wavelength is greater than that cited for the NRAO work, the source is much more favourably placed in the sky for observations at Sao Paulo. The atmospheric attenuation factor was comparable to the value cited for NRAO and varied between 1.2 and 1.5.

Table 1 Flux density of Cen A and Virgo A (Jy)

Date (1974)	$F_{V_{Cen}} \pm s_{Cen}$	$F_{V_{Vir}} \pm s_{Vir}$	$r \pm s$
April 30	19.7 ± 0.4	20.5 ± 0.5	0.962 ± 0.032
May 7	21.6 ± 0.7	20.9 ± 0.5	1.034 ± 0.041
May 14	21.5 ± 1.5	20.4 ± 0.3	1.056 ± 0.076
May 20	24.5 ± 0.6	24.3 ± 0.6	1.008 ± 0.034
June 3	21.6 ± 0.8	20.9 ± 0.1	1.031 ± 0.040
November 8	22.5 ± 0.9	20.6 ± 0.8	1.089 ± 0.059
November 28	21.9 ± 1.2	20.1 ± 1.0	1.089 ± 0.078
November 29	22.5 ± 0.6	24.7 ± 1.2	0.914 ± 0.052
December 6	20.3 ± 0.4	20.3 ± 0.3	1.000 ± 0.026

In Table 1 we also give the ratio r of the flux density of Cen A and Virgo A; the error s of the ratio is given by⁵

$$s^2 = s_{Cen}^2/F_{Vir}^2 + [(F_{Cen}/F_{Vir})^4 (s_{Vir}^2/F_{Cen}^2)]$$

A cursory inspection of the values of the ratio indicates that we have no evidence for variability at this wavelength during the times that these observations were made. A simple statistical analysis equivalent to the analysis given by Fogarty *et al.*⁶ confirms that there is no statistical significance for the suggestion that the source might have a variable flux density at 13.5 mm.

If, as it seems from our data and those of Kellermann¹, the source is variable at 3.4 mm but not variable at 13.5 mm, there must be some strongly wavelength-dependent radiation mechanism which enables the source to decrease its intensity at 3.4 mm with a very short time scale, while remaining constant at 13.5 mm.

We note that the flux density of this source is approximately constant, ≈ 22 Jy, over the wavelength range from 3.4 mm to 13.5 mm. So another interpretation of the observations is that the one measurement by Kellermann¹ of 14.0 ± 1.5 Jy on March 28, 1974, is in error and that the source is strong but not variable.

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Optical variation of 3C371

WHILE studying the nucleus of 3C446 Cannon and Penston¹ put forward a hypothesis involving a small continuous source of constant luminosity which may be temporarily obscured by absorbing clouds. This hypothesis is supported by our observations² of the nucleus of the N-galaxy 3C371, which reveal variations of its luminosity during a characteristic time of $t = 40$ min, or 2.4×10^3 s.

The light curve taken on September 12, 1972, resembles those of eclipsing binary stars (Fig. 1). M. I. Lavrov of the Kazan University (USSR) suggested (personal communication) that the luminosity changes seem to be similar to the luminosity variation of Algol. It may be supposed that QSO nuclei, the nuclei of Seyfert galaxies, the nuclei of N-galaxies and other compact extragalactic objects have two or more components rotating around a common centre of gravity. In this case the radius of these objects could be less than a limit given by

$$ct = (3 \times 10^{10} \text{ cm s}^{-1}) \times (2.4 \times 10^3 \text{ s}) \\ = 7.2 \times 10^{13} \text{ cm}$$

or approximately 5 AU.

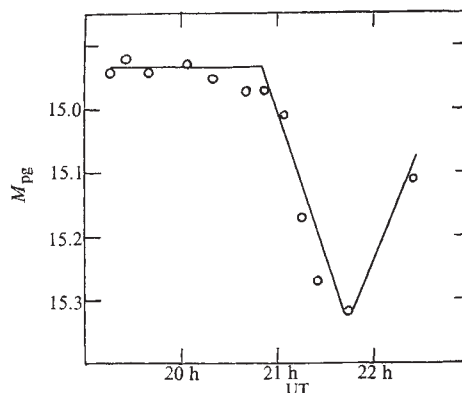


Fig. 1 Light curve of 3C271

Assuming that the velocity of these objects is less than the velocity of light, their dimensions could be in the range 10^{12} – 10^{13} cm, with a volume of 10^{36} – 10^{39} cm³.

Setting a typical mass as $5 \times 10^{10} M_{\odot}$ their density will be some 10^5 – 10^8 g cm⁻³.

Such objects may resemble the hyperdense bodies required by Ambartsumian's theory³ that galaxies and stars arise as a result of disintegration of compact objects.

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Galactic gamma rays from the superposition of X-ray sources

SATELLITE observations¹⁻³ show an enhanced flux of high energy photons (>30 MeV) along the galactic plane. The intensity has a broad and flat maximum extended $\sim 60^\circ$ around the galactic centre. The average value of the flux in the central region is $(1.1 \pm 0.3) \times 10^{-4}$ photons (cm² sr)⁻¹ in the anticentre direction it is about a factor of five less. The energy spectrum in the range (35-210) MeV is quite flat and possibly indicates that the gamma rays arise from decay of π^0 mesons.

Various models have been proposed to account for this flux in terms of collisions between cosmic rays and interstellar matter. On these models the observations of gamma rays would give important indication on the distribution of matter and cosmic rays^{4,5}.

The distribution of gamma rays also resembles the distribution of X-ray sources inferred from the third Uhuru (3U) Catalog. So the gamma-ray flux may result from the superposition of sources rather than having a diffuse origin. This idea is not new⁶⁻⁸ but the better quality of SAS-II observations compared with those of OSO-III and the larger extent 3U encouraged me to reconsider the problem.

Figure 1 shows the distribution of the counts from galactic compact X-ray sources and that of gamma rays observed by SAS-II and OSO-III. The distribution of the X-ray counts has been obtained by unfolding that relative to 3U sources with the same angular response function of SAS-II ($|b^{II}| < 10^\circ$) in the regions: $180^\circ < l^{II} < 50^\circ$, $160^\circ < l^{II} < 180^\circ$, and with the one of OSO-III ($|b^{II}| < 15^\circ$) in the region: $50^\circ < l^{II} < 160^\circ$. For variable sources I have assumed the

minimum flux (this is justified because of the small duty cycle of the flare activity). The width of the central maximum of the X-ray counts distribution is of the same order of magnitude as that reported by SAS-II for gamma rays. The flux of galactic gamma rays shows secondary peaks corresponding to regions where the X-ray sources are very powerful or very numerous. Nevertheless, this fit is inadequate because the ratios of the values of counts at various galactic longitude are very different for the gamma and X-ray distributions. A good agreement is obtained by considering only the steady sources (Fig. 2). This distribution does not take into account the sources 3U0620+23 and 3U1510-59 (possibly supernova remnants) and the anomalously strong (variable ?) sources 3U1636-53 (261 ± 3 counts s^{-1}) and 3U1735-44 (210 ± 6 counts s^{-1}). The source 3U1728-24 has also been excluded because it seems to be variable⁹.

The dashed line shows the gamma-ray distribution. The best agreement between the two distributions is obtained if one assumes that the sources emit equal amounts of energy in the X and gamma range. This requires the spectrum of the sources to be very hard (a hard spectrum is consistent with an extrapolation¹⁰ up to energy of 100 MeV of the gamma-ray emission data from Cyg X-2 in the range 1-10 MeV) or that there is an additional mechanism leading to gamma-ray emission. At present the models proposed for the X-ray emission suggest thermal sources with a temperature $kT \ll 100$ MeV. But flare-like emission at this energy could occur in the presence of strong magnetic fields with a particular configuration of the force lines. In this case protons could be accelerated to energies so high as to produce, in p-p collisions, neutral mesons which in turn decay in two gamma rays.

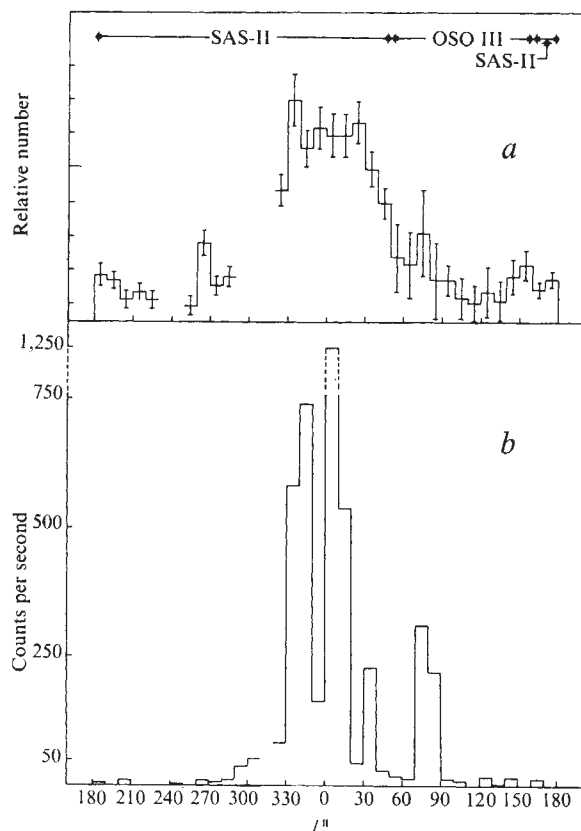


Fig. 1 *a*, Distribution of high energy (> 100 MeV) gamma rays along the galactic plane. *b*, Distribution of the counts from compact X-ray sources obtained by unfolding that relative to 3U sources with the same angular response function of SAS-II and OSO-III.

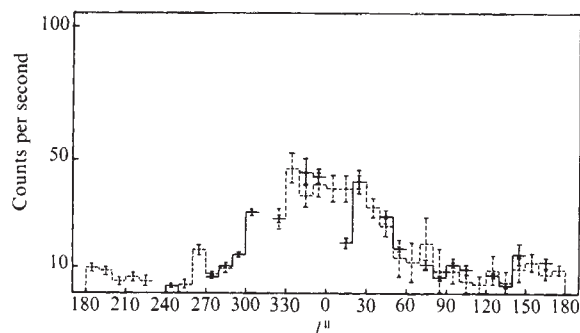


Fig. 2 —, Distribution of counts from steady X-ray sources; ---, distribution of gamma rays.

In my opinion it is, however, premature to conclude that the gamma-ray flux observed by SAS-II and OSO-III is the result of a superposition of sources rather than to cosmic ray collisions. Indeed, if X-ray sources are distributed along the spiral arms, their gamma-ray flux would have the same geometry as that resulting from cosmic ray collisions.

The model could be tested by searching for time variations in the gamma-ray counts (one can reasonably expect them if gamma rays arise from flares) and by obtaining gamma-ray data with better angular resolution to bring into evidence peaks in correspondence to individual X-ray sources.

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'Seasoning' of latent damage trails in lunar samples

CHARGED particle track analysis has been used extensively in the study of both terrestrial and lunar samples¹⁻⁵. In the latter, the tracks are produced by both internal and external sources. A careful analysis of etched track parameters not only helps in identifying various track-producing sources but also yields useful information about their charge and energy spectra. Most of the lunar samples analysed⁶ show that the track parameters of iron group nuclei from solar and galactic sources are very different from and more stable than those produced by (for example) 9.6 MeV per nucleon ⁵⁶Fe ions in simulation experiments in the laboratory. Similar effects are expected for other charged particle tracks. It seems as though the latent damage trails in lunar samples have been 'seasoned' or their structures have been so modified by lunar conditions (particularly, lunar temperature and high doses of light charged particles) that significant changes are brought about in their properties.

Thus, to interpret correctly the results of charged particle track analysis of lunar samples, one needs to study the

characteristic differences between naturally 'seasoned' tracks in lunar samples and 'unseasoned' tracks produced in the laboratory. Here we describe a study of the relative roles of temperature and time in the process of 'seasoning'. Attempts have been made to 'season' or to 'stabilise' the latent damage trails by annealing the samples under various combinations of temperatures and time intervals. We also give a comparison of the annealing properties of 'seasoned' and 'unseasoned' damage trails.

A big piece of tektite glass was exposed to thermal neutrons to produce induced fission of uranium atoms present in it as an impurity. The piece was then cut into a number of slices. On polishing and etching the slices, they were found to have a track density of about 10^6 tracks cm^{-2} . The uranium distribution in the glass was extremely uniform. The slices were then broken further into small parts, and some of them systematically annealed at temperatures of 300 °C, 500 °C and 550 °C for time intervals varying from 2 min to 1.6×10^4 min, 6×10^2 min and 3×10^3 min respectively. The purpose of these investigations was to study the track annealing properties of the glass and the characteristic differences between 'long term' and 'short term' annealings. The slices annealed at 300 °C for 1.6×10^4 min simulate the production of the 'seasoned' or 'stabilised' tracks; those annealed at 500 °C and 550 °C for 600 and 30 min respectively produce 'unseasoned' tracks. The loss in track density (expressed as a percentage of the unannealed track density) is given as a function of the annealing time in Fig. 1. To produce the same degree of annealing in samples exposed to different temperatures, they were to be annealed for such times that equal losses in track densities were obtained for all the samples. Figure 1 shows that to have a loss in track density of (say) 84% for the samples annealed at 550 °C, 500 °C and 300 °C, we need annealing times of 2.3, 9.3 and 1.6×10^4 min respectively. In practice, the sample annealed at 300 °C for 1.6×10^4 min (sample A) was kept for further experiments, while two new samples (B and C) were annealed afresh at this stage. Thus all the three samples contained fission tracks annealed to the same degree but in different modes: sample A contained 'seasoned' tracks while B and C contained 'unseasoned' tracks. All the three samples were annealed later at 300 °C for time intervals ranging from 2 min to 1.6×10^4 min, and the loss in track density as a fraction of the 16% tracks surviving after the first annealing step (Fig. 1) was plotted as a function of annealing time (Fig. 2). Although there is no appreciable difference in the residual track densities for shorter annealing times ($\leq 10^2$ min), the longer annealing times do show a marked difference in the stabilities of 'seasoned' and 'unseasoned' tracks—the 'seasoned' ones being the more stable.

Fig. 1 The annealing pattern of (neutron induced) 'fresh' fission tracks in a tektite glass as a function of the temperatures and time intervals used. ●, 550 °C; ○, 300 °C; ×, 500 °C.

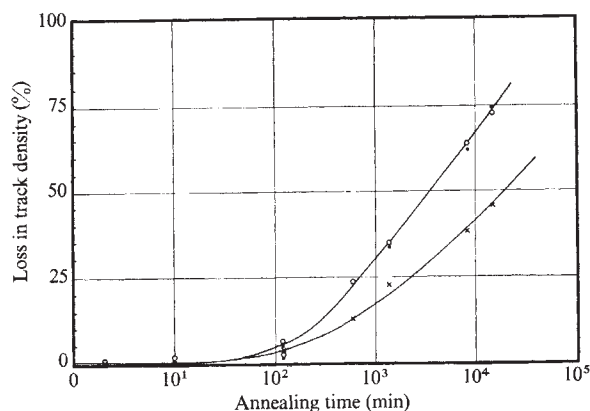
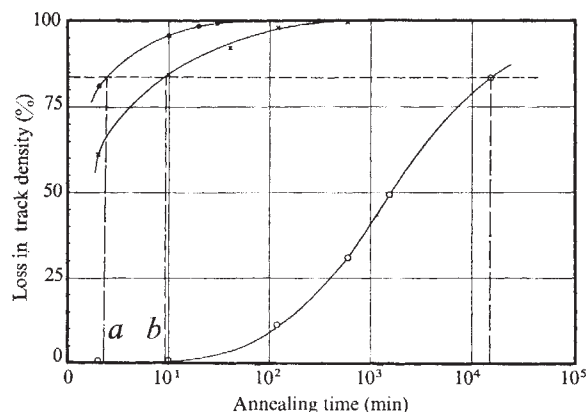


Fig. 2 The annealing behaviour of 'seasoned' and 'unseasoned' fission tracks in a tektite glass as a function of time at a fixed temperature of 300 °C. 'Seasoned' tracks are more stable. ●, 550 °C; ○, 500 °C; ×, 300 °C.

So annealing temperatures and their duration are important for the redistribution of the damage pattern during the 'seasoning' process. For example, a latent damage trail annealed at a low temperature for a long interval of time (conditions similar to lunar environment) readjusts itself to a more stable state than if it is subjected to a high temperature for a short interval of time. Thus, the degree of annealing is not the only important parameter in controlling the redistribution of the damage pattern, but the 'mode of annealing' plays an equally important role. This implies that the etched track parameters of 'seasoned latent damage trails' (as mostly found in lunar samples) and the 'unseasoned ones' (as obtained in the laboratory) will differ significantly. Their direct comparison is not justified and more work is necessary on the two types of tracks before any firm conclusions may be drawn.

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Molecular stratigraphy

ORGANIC molecules contained in sediments were called 'chemical fossils' by Eglinton and Calvin¹, and Calvin² has used the term "molecular palaeontology" to emphasise the biological heritage of those components. They recognised the important concept that molecular organic components in sediments may be viewed in much the same way as morphological remains of organisms. Others have rigorously examined this concept and solidified its basis^{3–6}. We report organic geochemical data on some Pleistocene sediments, correlated by physical stratigraphic methods, which indicate

Table 1 Samples and extraction data

Sample	Lateral distance* (km)	Dry weight (g)	Extractable proportion (%)†	Proportion of heptane eluate (%)
Mono Basin 1-L-76	2.4	17.5	4.70	0.79
1-L-76(10B)		15.4	3.84	1.97
Searles Basin Core B-Bed T2b	2.6	23.9	1.81	‡
Core C-Bed C-6		28.4	1.20	1.80
Core D-Bed D-4	3.4	31.8	0.87	1.38
Core E-Bed E-4		30.2	1.28	1.37

* Between stratigraphically correlated samples.

† On dry sample weight basis.

‡ Abundant sulphur (eluted with heptane fraction) irreversibly precipitated on concentration.

that the organic composition of sediments may be utilised as a correlation parameter comparable to morphological fossils in biostratigraphy. The samples we have chosen for study are lacustrine sediments from two Pleistocene basins of eastern California.

Mono and Searles Lakes are two of several strongly alkaline desert lakes of eastern California. Mono Lake is in a closed drainage basin, with neither an outlet to the sea nor a sink. The lake is now desiccating⁷⁻¹⁰, although it is not yet sufficiently concentrated to deposit salts. Mono Basin contains a sequence of Pleistocene lacustrine silts and diatomites approximately 1.5 km thick^{8,11}. Arching of the

bottom of the lake, forming Paoha and surrounding islands, occurred late in the development of the basin^{8,12}. Detailed stratigraphic columns of Late Pleistocene sediments, given the informal designation Paoha Island Sequence, were measured on Paoha Island. The sediments are generally finely-laminated algal silts, diatomaceous silts or silty diatomites, with some sands through the section, and rare basaltic and rhyolitic tuffs¹³. The samples from Mono Basin were collected from measured stratigraphic sections 2.4 km apart. Correlation of the two measured sections is based on lithologic sequences.

Searles Lake is the dry vestige of one of a chain of four Pleistocene Lakes^{14,15}. Searles Basin contains a remarkable sequence of Pleistocene salts and muds, approximately 1 km thick¹⁶; salts and brines from the basin are being mined on a large scale. Kerr-McGee Chemical Corporation donated four core segments (cores B, C, D, E) of the Parting Mud¹⁴; cores B and C were drilled 2.6 km apart, and cores D and E 3.4 km apart. The sediments consist primarily of finely laminated clays, occasionally with intercalated thin laminae of various carbonate minerals. The presence of aragonite, dolomite, pirssonite and gaylussite has been documented by X-ray diffraction. Large (>2 cm), apparently diagenetic crystals of pirssonite and gaylussite, oriented by their 'c' axes at high angles to bedding, are common in parts of these cores. We found no sand in the section studied. Stratigraphic correlation between drill cores is based on thin (2-3 mm) rhyolite tuffs, now largely zeolitised to phillipsite, which occur near the top of the Parting Mud.

We used a mixture of benzene and methanol (3:1 v/v) to extract the organic components from dried, powdered sediment samples. The concentrated extract was fractionated by liquid-solid chromatography on silica gel, successively eluting with heptane, 5, 10, 15 and 20% (v/v) ethyl acetate in heptane, ethyl acetate and methanol. The heptane eluate (nC₇ fr.) was analysed by gas-liquid chromatography (GLC). After initial GLC analysis, the heptane eluate was segregated further into three component fractions using silver nitrate impregnated thin-layer chromatographic plates (silica gel G, 0.25 mm, 15% AgNO₃ in methanol, mobile phase heptane-benzene=99:1). After recovery each of these three fractions was again analysed by GLC, with authentic compounds coinjected. The final analytical procedure was combined gas chromatography-mass spectrometry (GC-MS) on a DuPont 21-491 mass spectrometer.

For this work we have sampled time-equivalent strata at widely separated locations. Mono Basin samples 1-L-76 and 1-L-76(10B) represent exact lateral equivalents; in the Searles Basin samples, Bed T2b (Core B) is the exact lateral equivalent of Bed C-6 (Core C), similarly Bed D-4 (Core D) corresponds to Bed E-4 (Core E). The total proportion of extractable material in a sediment and the concentration of hydrocarbons in that extract has been used in the past to judge similar organic compositions¹⁷. Our extraction data (Table 1) for precisely correlated beds within a sedimentary sequence indicate that this criterion may be uncertain.

Stratigraphic variability in organic composition for sediments from Mono Basin has already been documented^{13,18}. Figure 1 shows that sediment samples of the same bed, collected from localities 2.4 km apart, possess an identical distribution of individual molecular components. Phytane (2,6,10,14 tetramethylhexadecane), nC₁₇, nC₂₇, and nC₂₉ are the dominant compounds in the hydrocarbon fraction of these samples. The homologous series of *n*-alkanes has an attendant homologous series of mono-olefins, one of which is labelled (1Δ-nC₁₆) on the figure. Peak a represents a composite of two components, one an unidentified branched alkane, the other being 1Δ-nC₁₈. Identification of peaks b, c, x, y, z is still unsubstantiated.

Figure 2 also illustrates lateral continuity in organic composition; although two stratigraphic horizons in Searles Basin have different compositions, each unit laterally ex-

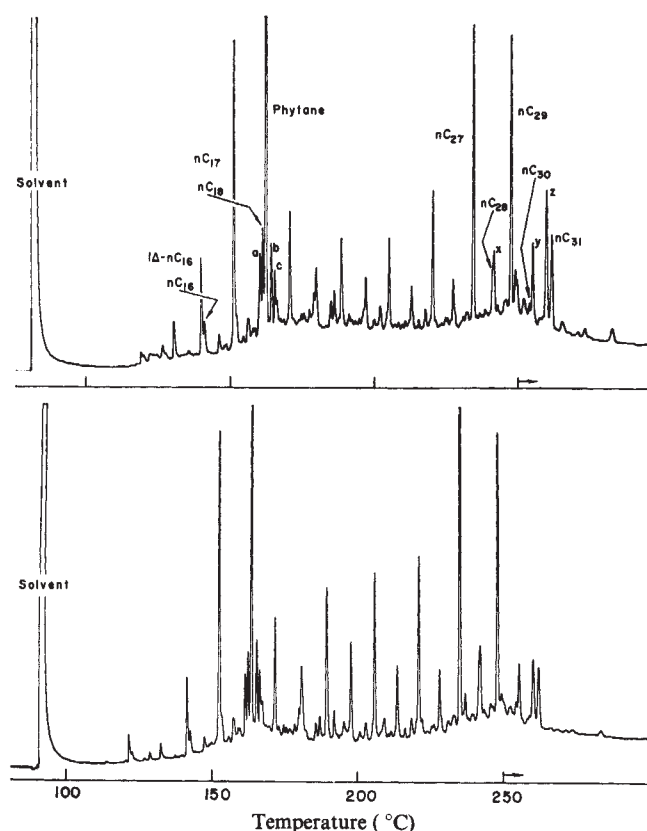


Fig. 1 Gas chromatograms (nC₇ fr.) of time equivalent sediment samples, Mono Basin, California. The two samples were collected from measured stratigraphic sections located 2.4 km apart. (Analysis was on a 15 m × 0.5 mm support coated open tubular column, coated with SE-30, helium carrier gas flow 4.6 ml min⁻¹, temperature programmed 80-250 °C at 2 °C min⁻¹, injector temperature 275 °C, detector temperature 295 °C.)

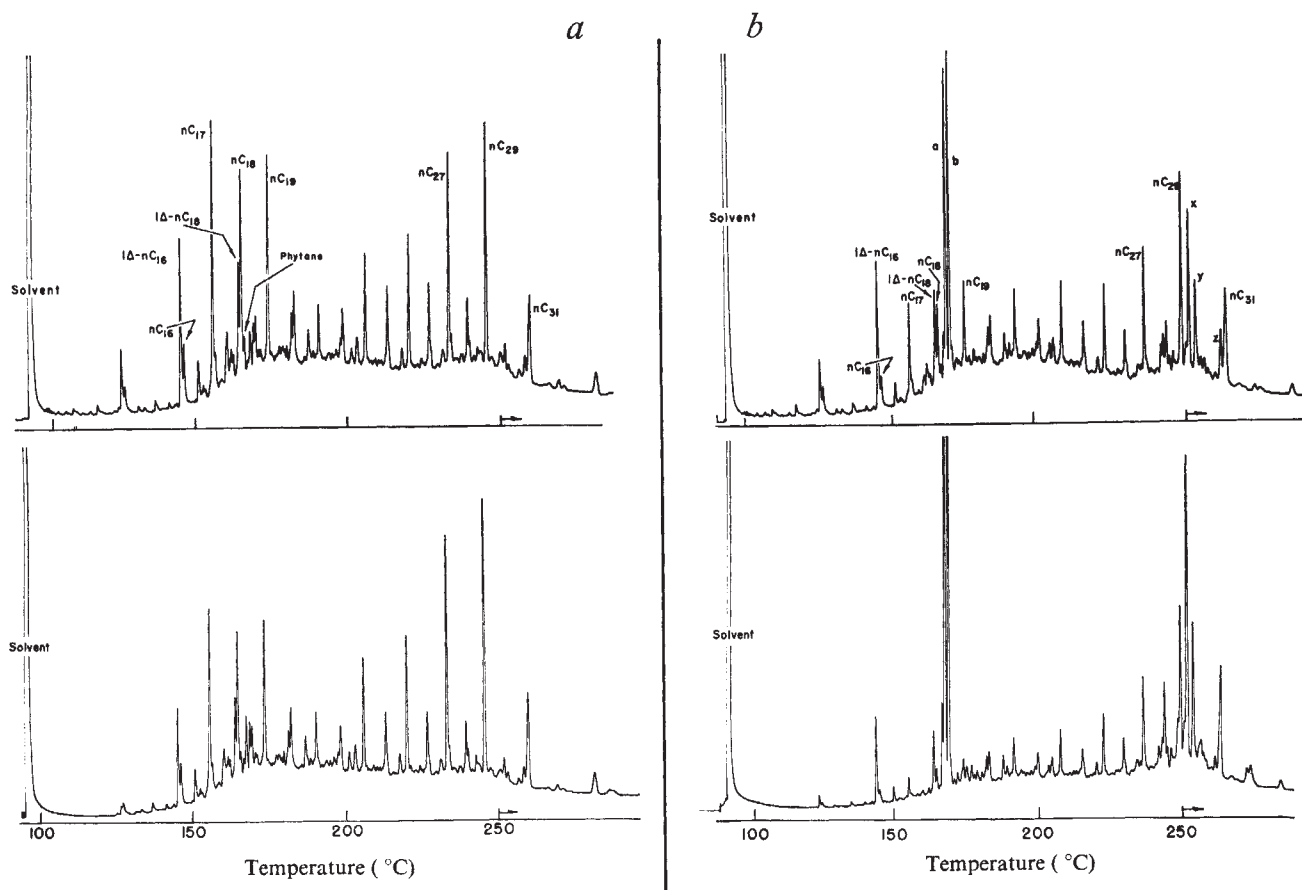


Fig. 2 Gas chromatograms (nC_7 fr.) of time equivalent sediment samples, Searles Basin, California. Analytical conditions as for Fig. 1. a, Bed T2b (core B) is the exact time equivalent of Bed C-6 (core C), the core locations are 2.6 km apart. These samples represent the interval 1–4 mm below Tuff II. b, Bed D-4 (core D) is the time equivalent of Bed E-4 (core E), the core locations are 3.4 km apart. These samples represent the interval 778–790 mm above Tuff I.

hibits a consistent composition. The series of mono-olefins observed in the previously discussed samples is more prominent in these sediments from Searles Basin. For the stratum correlated between cores D and E, the two most abundant constituents (Fig. 2b, peaks a and b) are mono-olefinic isoprenoids (peak a: 3,7,11,15 tetramethylhexadec-1-ene; peak b: 3,7,11,15 tetramethylhexadec-2-ene). The mass spectra for these two components were identical, assignment of the double bond position is based on GLC retention times¹⁹. Identification of peaks x, y, and z is still unsubstantiated; peak x seems to represent a mixture of two $C_{28}H_{46}$ sterenes, and peak z a mixture of two $C_{30}H_{50}$ pentacyclic triterpenes.

Therefore we agree with Eglinton and Calvin^{1,2} on the fossil nature of some organic molecules in sediments. We suggest the new term 'molecular stratigraphy', to convey the qualification that not only are organic molecules inherited from biota, but that these molecules must be constrained by biostratigraphic principles such as facies. Lateral variation in the molecular organic composition of a stratigraphic unit may result either from ecologically controlled distributions of biota, or from selective preservation (over- or under-representation of components depending on the character of the material being fossilised with respect to depositional regime). The lacustrine sediments we have examined in this study grade laterally into alluvial sedimentary sequences. The organic composition of the alluvial facies no doubt will be different from that of the lacustrine facies. In this work we selected time-equivalent samples

representative of the same depositional facies. In sedimentary sequences where fossils are scarce, molecular stratigraphy may represent the only correlation parameter available.

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Electron spectroscopic observations on extreme pressure lubrication

COMPOUNDS of sulphur, phosphorus, zinc and chlorine are added to lubricating oils to control wear under arduous conditions by forming films on rubbing surfaces. The films are extremely thin and frequently amorphous. Only rarely has it been possible to identify compounds present although electron probe microanalysis can show the distribution of elements. We here present new information, obtained from electron spectroscopic measurements, mainly concerning the binding energies of photoelectrons (ejected with $AlK\alpha$ X rays) the magnitudes of which are characteristic of particular atoms and electrons, and which may therefore be used for analytical purposes. We also made some observations of the kinetic energies of Auger electrons, which are also well defined and of analytical value. These energies depend on the charge on the atom, so that for states of combination of an element where the charges on the atoms are sufficiently different, valuable 'chemical shifts' are observed; sulphur is a favourable case with a shift of about 6 eV from sulphide to sulphate. Only electrons that escape without collision are of value, so the analysed depth was limited to a few tens of Å, the region of interest in lubrication.

Specimens of EN31 steel, after diamond polishing and scrupulous solvent cleansing, were treated in solutions of additives in non-polar white oil. They were either statically immersed for 16 h at a temperature of 145 °C, or rubbed for 10 min against a disk of the same material in a wear machine. Before examination in the electron spectrometer the specimens were washed in non-polar solvents to remove oil.

The surface of a specimen immersed in a solution of elemental sulphur in white oil showed S2p peaks, denoting binding energies of 168.8 and 164 eV. The first peak value was greatly diminished by washing the specimen in water; it matched that of a thin layer of sulphate on iron. The second value was reasonably close to that obtained for elemental sulphur. The peak value was not, however, much reduced by washing in carbon disulphide, so reservations must be made in attributing it to sulphur. The rubbed specimen showed much less sulphate but gave a large signal corresponding to sulphide, and part of the surface oxygen was seen from the oxygen 1s spectrum to correspond to oxide. We conclude that whereas the specimen which had been immersed had a rather uniform coating of iron sulphate, the surface of the rubbed specimen was at least partially oxidised and carried a relatively thick but discontinuous layer of iron sulphide.

A specimen heated in a solution of dibenzyl disulphide in white oil gave peaks in the same positions as the sulphur solution. Again, that which indicated a binding energy close to 169 eV was very sensitive to washing in water, and we therefore attributed it to sulphate. The interpretation of that at approximately 164 eV is less straightforward in that the additive itself gave a very similar binding energy. We found, however, that dibenzyl disulphide could be removed by the solvent washing. It was also sensitive to washing in water unlike the substance on the specimens. The peak at 164 eV cannot, therefore, be attributed to dibenzyl disulphide. Dibenzyl monosulphide, a proposed intermediate¹⁻² which has a closely similar binding energy, can be ruled out because it volatilises in the vacuum of the spectrometer. Benzyl mercaptide can also be ruled out because it would give a peak in the ferrous sulphide position. As in the previous experiment, it is tempting to assign this peak to sulphur, but there must be reservations.

The specimen rubbed in the white oil solution of di-

benzyl disulphide also behaved very like that in the sulphur experiment, showing mainly sulphide sulphur, with some sulphate, and oxygen present in part as oxide.

Most of our work has, however, been done with a commercial zinc dialkyldithiophosphate additive in which the alkyl groups are predominantly methylpentyl. This additive contains 13% mineral oil. Pure zinc di-*n*-butyldithiophosphate was also examined.

Specimens immersed in solutions of the commercial additive in white oil had zinc, phosphorus and sulphur on the surface; the sulphur was in two forms, the more abundant of which corresponded to sulphate. The zinc, phosphorus and the minor sulphur component displayed electron binding energies indistinguishable from those of the original additive. The binding energy of that sulphur was also the same as that of sulphide sulphur. Electron spectroscopy alone, therefore, could not distinguish between the two possibilities that the minor sulphur was present as a sulphide film or was present together with the zinc and phosphorus in a layer of adsorbed additive. The second possibility was eliminated by re-examining the specimens after they had been washed in boiling acetone. This treatment, which supposedly³ removed adsorbed additive, did not change the spectrum. Although the possibility that a sulphide film is present is not completely out of the question it seems more likely that the minor sulphur is bound up, together with the zinc and phosphorus, in a decomposition product of the original additive. The evidence for this is the relatively small variation of the atomic ratios of zinc : phosphorus : sulphur found in our experiments over a range of conditions. This ratio is, for example, 1 : 1 : 0.6 at 140 °C, whereas that for the original additive is 1 : 2 : 4. Zinc phosphate, zinc sulphide and zinc sulphate were not present and the presence of zinc oxide also seems unlikely from a consideration of Auger line widths. These observations, together with others not reported here, suggest that the reaction mechanism varies with temperature and that a complex, possibly polymeric, phosphate forms on the metal surface on which there is also iron oxide and possibly iron sulphide or sulphate. The pure di-*n*-butyl compound gave much less sulphate on the surface, which suggests that in the commercial additive either impurities which contained sulphur or sulphur contained in the mineral oil carrier contributed largely to sulphate formation.

As with the sulphur additives, the specimens rubbed in the solution of the commercial zinc dialkyldithiophosphate showed a much smaller sulphate peak, but otherwise the spectra were rather similar to those of the immersion specimens. The atomic ratios of zinc, phosphorus and sulphur showed less variation and more of the surface was iron oxide.

Other investigators have suggested that it is possible, under various conditions and at various temperatures, that adsorbed additive, iron sulphide and iron benzyl mercaptide are responsible for the anti-wear performance of the dibenzyl disulphide (refs 1 and 2, and R. C. Coy and T. F. J. Quinn, unpublished). The literature on zinc dialkyldithiophosphates proposes many reaction schemes, often unsupported by analytical evidence, in which zinc oxide, zinc sulphate, zinc phosphate and polymers with definite zinc-phosphorus-sulphur ratios of 1 : 2 : 4 to 1 : 2 : 2 are postulated (see refs 3-5).

The methods of electron spectroscopy clearly provide new opportunities for progress in the study of lubrication phenomena. In particular, they have given information on very thin films on rubbed surfaces—films which have been too thin to examine by other means. Additionally, by showing large differences in the compositions of films on immersed and rubbed specimens, they indicate the dangers inherent in basing reaction mechanisms on immersion tests alone.

A fuller account of this work will be published elsewhere.

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Stable cold cathode arc

THE cold cathode arc, so called because of the very high current densities and low temperatures observed at the cathode, has been subjected to intensive theoretical and experimental study¹⁻⁴. Many theories of the 'cathode spot' have been proposed, but distinguishing between them experimentally has proved to be a formidable task, because of the rapid and unstable motion of the spot, and the large pressure and temperature gradients existing within a very short distance of the cathode surface.

It is possible that a caesium discharge run at temperatures up to 1,200 K with a tungsten cathode, and with the entire discharge (not just the electrodes) heated externally, may behave as a stable, cold cathode arc. The low ionisation potential of caesium (3.9 V) could allow enough plasma by thermal ionisation for measurable currents to be obtained, and the work function of tungsten (4.5 eV) may be high enough for cold cathode behaviour to occur.

The discharge tube consisted of a commercial, high pressure, sodium arc lamp, with the arc tube filled with 0.1 g of caesium instead of sodium. The arc tube was made of 8 mm diameter alumina, with a space of 9 cm between tungsten electrodes of 1 cm² surface area. There was a silica-glass outer tube, and the space between the tubes was filled with argon at a pressure of 200 mm Hg. The device was enclosed in a cylindrical furnace capable of producing temperatures of up to 1,400 K, with an adjustable temperature control, and with its axis inclined at about 20° to the horizontal. Tungsten wires to the electrodes of the device were brought out of the furnace in thin, sealed, silica-glass tubes. Temperatures of up to 1,100 K were achieved without damage.

Before measurements were taken, the position of the arc tube relative to the temperature gradient along the furnace (higher temperature at the high end) was adjusted until liquid caesium was observed to condense only at the colder end of the arc tube. The temperature of the caesium gas was taken to be that measured by a thermocouple near the hotter end of the tube. The temperature of the caesium liquid (which controlled the gas pressure) was estimated to be 5% lower (Kelvin) than the gas temperature, which corresponded with the temperature measured by a thermocouple near the colder end of the tube.

A typical plot of current against voltage (Fig. 1) shows that for both current directions, there is a proportional increase of current above about 8 V, indicating a constant electrode voltage, and a constant arc column resistance. The slope of the straight line (Fig. 1) gives the resistance of the column, and its intercept on the voltage axis gives a measure of the electrode voltage. The electrode voltages obtained when the colder electrode was used as cathode were discarded, as they tended to vary (presumably depending on the amount of contact between the tungsten electrode and the liquid caesium). The electrode voltages obtained when the hotter electrode was used as cathode, however, were reliable and repeatable and the shapes of the curves on initial application of voltage

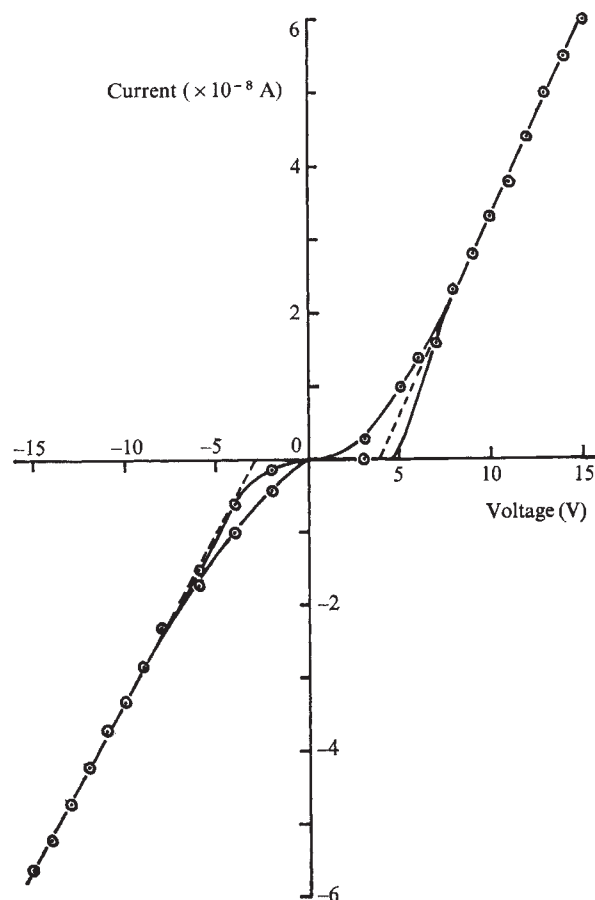


Fig. 1 Typical plot of current against voltage. Gas temperature, 760 K. Positive readings for hotter electrode as cathode.

(Fig. 1, the lower curve) and on subsequent applications (Fig. 1, the upper curve), seemed to rule out thermionic emission.

The electrical conductivity, σ , of the caesium column was derived from the resistance measurements. Log σ was then plotted against the reciprocal of the gas temperature, T ; the result was a straight line (Fig. 2). The electrode voltage obtained when the hotter electrode was used as cathode was also plotted against $1/T$ (see Fig. 3). It was found that the experimental points could be fitted to an exponential curve (Fig. 3) with an asymptote at 2.65 V.

Above a gas temperature of about 970 K (~ 1 atm gas pressure), no arc could be obtained, and the current dropped below the measuring limit of the apparatus ($< 10^{-10}$ A). Between about 770 and 970 K, the arc could be made to run, and when it did run it gave repeatable results, but it showed a tendency to extinguish or not to start at all. Below about 770 K the arc would always run.

The standard formula for the electrical conductivity of a weakly ionised gas is $ne^2/n_a mcq$, where n is the plasma number density, n_a the gas number density, and e , m , c and q , respectively, the electron charge, mass, thermal speed, and collision cross section for momentum transfer with neutral particles. Assuming thermodynamic equilibrium conditions, Saha's equation may be applied⁵ to yield $n^2 n_a \exp(-e\bar{V}_i/KT)$, where $\bar{V}_i$ is the effective ionisation potential, and the dependence of the constant of proportionality on temperature can usually be neglected in comparison with the rapid variation of the exponential term with temperature. Thus, the electrical conductivity should obey a relationship of the form:

$$\sigma = \sigma_0 \exp[-e(\bar{V}_i - V_{\text{evap}})/2KT]$$

where $n_a \alpha \exp(-eV_{\text{evap}}/KT)$ is used, with V_{evap} increased by

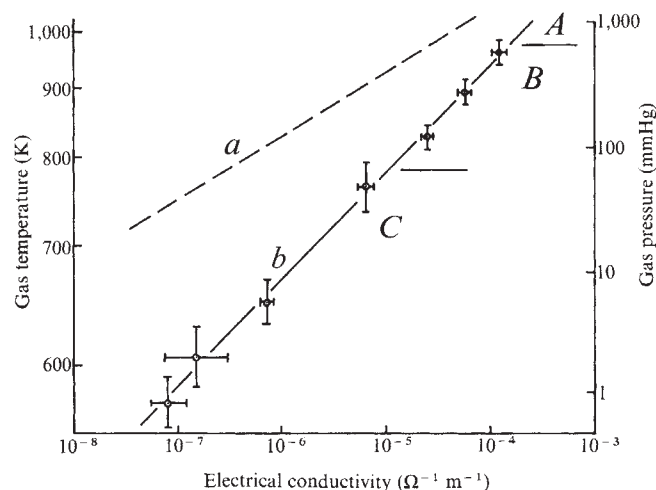
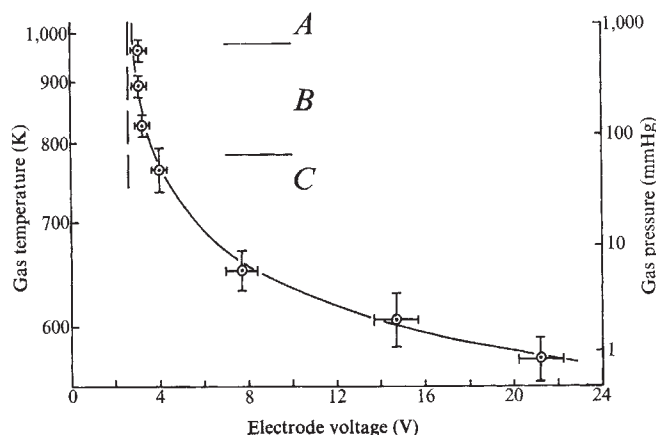


Fig. 2 Relationship between gas temperature and pressure and electrical conductivity. *a*, Theoretical line assuming no wall/electrode excitation mechanism *b*, experimental line. *A*, region of no arc; *B*, region of marginally stable arc; *C*, region of stable arc.

5% from its standard value, to allow for the fact that the caesium liquid temperature is 5% below the gas temperature.

The dashed line in Fig. 2(*a*) is obtained by substituting $\bar{V}_i = V_i = 3.9$ V and $q = 1.5 \times 10^{-14}$ cm² into the formula for σ . Clearly, straightforward volume ionisation is not the dominant mechanism. The experimental line (Fig. 2*b*) corresponds to $\bar{V}_i = 2.64$ V and $q = 4.10^{-12}$ cm², with estimated errors of ± 0.2 V on $\bar{V}_i$ and up to a factor of 3 on q . This result is readily interpreted in terms of a dominant mechanism involving excitation at the walls or the electrodes of the discharge. The lowest excitation potential of caesium, V_{exc} , is 1.4 V, so that the voltage required to ionise excited atoms is $(V_i - V_{exc}) = 2.5$ V, agreeing with the measured $\bar{V}_i$ within the experimental accuracy. In fact, at these temperatures, a very long time is required² to achieve volume thermal ionisation, so any appreciable wall or electrode mechanism could be expected to dominate. Furthermore, the very large experimental cross section (even allowing for the large possible error and the fact that the gas is caesium) provides a strong indication that the neutral particles are likely to be excited atoms or molecules.

Fig. 3 Relationship between gas temperature and pressure and electrode voltage. Experimental points are for the hotter electrode as cathode. The smooth curve is an exponential curve fitted to the experimental points, with asymptote at 2.65 V. Regions A–C as for Fig. 2.



The asymptotic value of electrode voltage, 2.65 V (see Fig. 3) is also consistent with an explanation in terms of excited atoms. Allowing for a small drop in anode voltage ($\sim KT/e$), the asymptotic cathode voltage is again very close to $(V_i - V_{exc})$. Robson and von Engel⁶ have proposed a mechanism which seems to account for these observations. Excited atoms impinging on the cathode release secondary electrons with a high yield. The net energy required for an electron to escape is $e(V_i - V_{exc})$, and this determines the cathode voltage required.

The exponential curve fitted to the electrode voltage readings in Fig. 3 may be expressed in terms of gas number density

$$V = 2.65 [1 + (n_0/n_a)^{2/3}]$$

where $n_0 = 4 \times 10^{17}$ cm⁻³. In terms of the interparticle separation distance for neutrals $\lambda_a = n_a^{-1/3}$, this becomes

$$V = 2.65 [1 + (\lambda_a/\lambda_0)^2]$$

where $\lambda_0 = 1.4 \times 10^{-6}$ cm. Now λ_0 is about twice the scale length for the Schottky inverse square law image force acting on electrons associated with the surface potential barrier. Thus, the experimental result implies that when the neutral interparticle separation distance increases to a value comparable to the range of the surface potential barrier, more cathode voltage is required to pull electrons clear of the surface. This again is entirely consistent with the Robson-von Engel explanation.

What is the reason for the arc failing to run above 970 K? One possibility is that volume ionisation and thus (by the principle of detailed balancing) volume recombination become more important than surface effects above this temperature, and the discharge reverts to full volume equilibrium. But the dashed line in Fig. 1 suggests that even if that were to occur there should still be an easily measurable current. It would also be necessary for a volume mechanism for quenching excited states to become important². That would suppress the emission process suggested by Robson and von Engel and the current would then drop to the thermionic emission level for tungsten, below the minimum that could be detected.

Finally, a 'unipolar' arc effect was also observed. When the arc was run for long periods (30 min or so) in one direction, it could be observed to charge up, just like a battery. It would then deliver current to a resistive load for a comparable period. The 'e.m.f.' associated with the effect was about 1.8 V. This may have been caused by a monolayer of caesium ions adhering to the tungsten electrode, as has been observed by Langmuir and coworkers⁶ at much lower pressure, but if so, then it must have been formed at the anode during the charging process, and not at the cathode. If it had been formed at the cathode, it would have caused current to continue in the same direction after removal of the supply voltage, contrary to what was, in fact, observed.

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Electromagnetic effects at metallic fracture

JUST at the instant of tensile fracture of low-carbon iron specimens a transient magnetic field of the order of 1,000 gauss is produced¹. This phenomenon is observed even in the absence of any external magnetic field. Only the surface region of the fractured pieces are magnetised, with the fractured ends exhibiting distinct and opposite polarities. The magnetisation of each piece varies along the length, with maximum at the fractured end. Moreover, the induced magnetisation is retained for about 15 d in larger specimens and then decays almost exponentially. Iron specimens fractured under impact forces did not show any magnetisation. I now report observations of the complementary phenomenon of generation of an electric field at the instant of tensile fracture of metallic specimens, ferromagnetic as well as non-ferromagnetic.

Metals investigated, were 0.15% carbon steel, aluminium (4.5% Si, 0.5% Fe, 95% Al), brass (63.5% Cu, 0.1% Pb, rest Zn), zinc (99.67% pure) and lead (99.43% pure). The ultimate tensile strengths of these materials were of the order of 39.0, 29.4, 33.2, 6.2 and 2.0 kg mm⁻², respectively. The zinc and lead rods were processed by metal mould casting and the others by hot rolling.

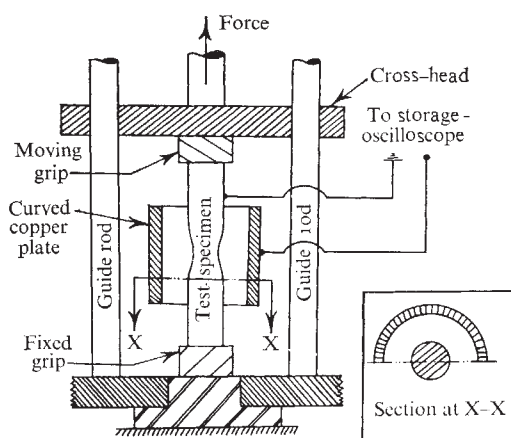


Fig. 1 Schematic diagram of the force application system of the tensile testing machine.

External electrical and magnetic disturbances (including the Earth's field) were shielded out during the experiments. Test specimens were stressed in vertical and horizontal directions, and also in different orientations with respect to the magnetic meridian. A coaxial, semicylindrically curved copper plate (30 mm internal diameter and 50 mm long) was used as an electric field sensor. A Hewlett Packard variable persistent storage oscilloscope was used to record the phenomenon. Measurements of potential difference were also made with the help of a d.c. microvoltmeter. The potential pickup wire soldered on to the outer surface of the curved copper plate was connected to the storage oscilloscope and the ground wire, first direct on to the surface of the test specimen with the machine body grounded separately and then with the ground wire of the oscilloscope connected to the machine body without disturbing the test specimen, the latter being at ground potential by contact with the machine parts. The curved copper plate did not suffer any mechanical jerk resulting from stressing and fracture (Fig. 1).

Test specimens had dimensions 9 mm diameter $\times$ 160 mm long and were fractured under tension in a horizontal Housfield Tensometer. The test specimens were machine turned to a

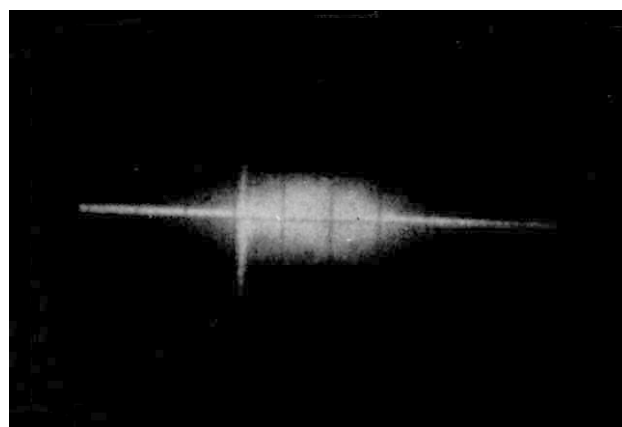


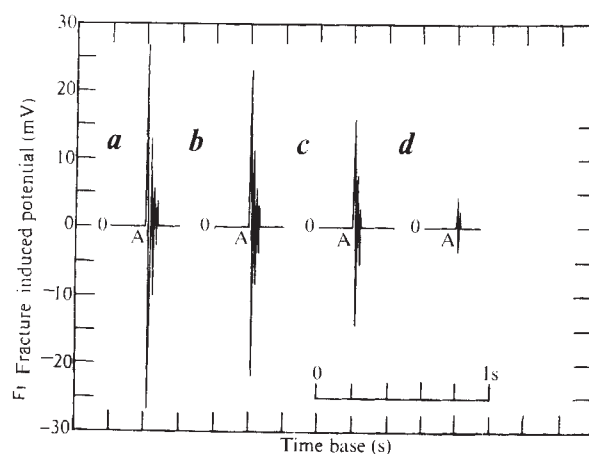
Fig. 2 Photograph of the voltage against time plot obtained on the storage oscilloscope for an aluminium specimen.

diameter of 7 mm in the central zone (Fig. 1) to ensure fracture in the centre. The transient magnetic fields in ferromagnetic specimens at the instant of tensile fracture were measured simultaneously as in my earlier work¹; the results agree with those of that study.

Figure 2 is a photograph of the voltage time plot obtained on the oscilloscope during the tensile stressing of an aluminium specimen. It shows the generation of an electric field just at the instant of fracture. The voltage developed seems to be oscillatory in nature, marked by rapid damping. The diffused light in the vicinity of the horizontal time axis in the photograph is caused by the trace-blooming effects of the oscilloscope beam because of its excessive intensity. Similar curves were obtained for all metallic specimens (steel, aluminium, brass and zinc) but the amplitudes of the voltage generated were different for different materials. Figure 3 shows the tracings of the curves obtained on the storage oscilloscope for different materials, both qualitatively and quantitatively. The nature of the curve is independent of the orientation of the specimen. Expanding the time scale on the oscilloscope was not achieved because of the difficulty of synchronisation of the oscilloscope triggering to the instant of fracture. An expanded time scale would probably have exhibited the phenomenon more clearly.

Results with lead rods were of particular interest. Rapid fluctuations of electrical potential difference of the order of $\pm 40 \mu\text{V}$ (peak value) occurred as soon as the cross-sectional area of the lead specimen started reducing rapidly, instead of a first sharp peak value at the instant of fracture as in other cases. This may be because lead is very soft, plastic in nature and

Fig. 3 Tracings of plots obtained for: a, carbon steel; b, aluminium; c, brass; d, zinc. A, Denotes instant of fracture.



under uniaxial tension it suffers a rapid decrease in cross-sectional area leading to a point fracture. Moreover, the number of current carriers per atom based on a free electron model for lead is very high².

The twin phenomena of the generation of electric and magnetic fields at the instant of tensile fracture basically represent the electromagnetic effects of mechanical force fields in metals. Influencing parameters are likely to include: breaking stress, number and nature of current carriers per atom, electrical and magnetic structures of molecules of metals, mode of fracture, and crystal lattice structure. Furthermore, fracture phenomena are predominantly influenced by dislocations in dynamic state and energised shock waves. The observed electric and magnetic effects might be attributed to electron-dispersion analogous to the radiation of sound by dispersion of elastic vibrations of the crystal lattice as a result of dislocations³.

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A new approach to improvement of yam *Dioscorea rotundata*

EFFORTS to improve the yam, *D. rotundata*, through hybridisation have been frustrated by the low rate of flowering, the very low rate of fruit setting and poor seed germination¹⁻³. These constraints have confined the availability of genetic resources for selection to the small number of cultivars which have been vegetatively reproduced from time immemorial. This continued vegetative propagation and the lack of hybridisation have precluded the possibility of introduction of new genetic characteristics, resulting in a stagnation of yam improvement. To facilitate yam hybridisation, we have re-examined the problems of flowering and seed germination. We first reported⁴ yam seed germination in November 1973, and Doku stated

that he had germinated yam seeds of *D. rotundata* (unpublished). Here we report the feasibility of germinating yam seeds, the rapid improvement of flowering, and the opportunity this development affords for expanding present germ plasm collections, thus facilitating yam improvement.

After a series of unsuccessful attempts, we obtained good germination in early 1973 when two important limitations for seed germination became evident⁵. First, the majority of the seeds were found to lack embryos or endosperms and second, they were found to possess a dormancy period of about 3 months after harvest. Having discovered these two limitations, seeds that seemed to contain embryos were selected and stored at room temperature until the end of the dormancy period. Seeds were sown in Petri dishes in March 1973, and in about 3 weeks more than 50% of the seeds germinated. After germination and the appearance of the first leaf, some 600 seedlings were transplanted into small pots and a month later, were transferred to larger pots and grown in the greenhouse. Tuber formation began about a month after transplanting. Flowering of some plants began in July 1973 with all the flowering plants bearing staminate flowers. None of the plants had produced any pistillate flowers by plant maturity. The plants showed a great deal of variability with respect to leaf shape, size and colour, canopy structure, stem colour and shape, plant height, and tuber shape and size. Tubers were forced to maturity by gradual decrease in watering and were harvested in February and March 1974, at which time most of the tubers weighed more than 500 g, which contrasts markedly with the observation of Waitt², whose material took 2 yr to produce a 450 g tuber from seed.

On April 1, 1974, each tuber was divided into several 'seed' (vegetative) pieces and planted in the field. The purpose of this second planting was to facilitate maximum growth, flowering and fruiting under field conditions and to provide at maturity, seeds to produce plants of a second generation in 1975. Throughout the growing season, data were collected on all phases of plant growth characteristics with special attention to date and degree of flowering and plant sex. Male plants began to flower in early July while female plants began to flower in late July and early August.

It was observed that the percentage of flowering plants, particularly females, was greater in the population originating from seeds than in those originating from tuber cuttings obtained by continuous vegetative propagation (Table 1). The degree of flowering per plant had also increased. Whereas plants grown from tuber cuttings usually produce less than 200 pistillate flowers per plant, plants originating from seed produced from 500 to 11,000 pistillate flowers. Furthermore,

Table 1 Comparison of the degree of flowering and plant sex between plants of *D. rotundata* originated from continuous vegetative propagation and from seeds

	Flowering (%)	Non-flowering (%)	Flowering plants (%)		
			Male	Female	Monoecious
Plants from continuous vegetative propagation	47.1	52.9	69	31	0.0
Plants from tubers originating from seeds	72.3	27.7	53.6	41.7	4.7

Table 2 Seed germination and seedling establishment of eight Nigerian yam populations

Parent and source	No. of seeds tested	Germination (%)	No. of seedlings transferred to field	Seedling survival (%)
Ihobia (IITA)	330	90.0	89	43.8
Iwo (Kwara State)	2,175	99.3	1,385	88.2
Laoko (IITA)	50	—	9	100.0
Mixed (IITA)	360	—	37	100.0
Okunmodo (IITA)	260	92.4	58	34.5
Umudike (East Central State)	1,269	98.5	977	85.9
Unknown	230	—	12	100.0
Wild (IITA)	500	—	416	100.0
Total	5,147	95.1	2,983	95.7



Fig. 1 Degree of yam flowering and fruit set. *a*, Male plant; *b*, female plant at the fruiting stage.

4.7% of the plants derived from seeds, produced both staminate and pistillate flowers on the same plant (monoecious), which is an important departure from the usual production of either staminate or pistillate flowers on separate plants (Table 1). This quantitative and qualitative change in the flowering habit of plants of the first generation presumably resulted from our conscious selection for flowering through propagating plants from seeds. Since the low degree of flowering has been one of the major limiting factors in seed production, it is expected that after several generations the degree of flowering will increase to an extent such that it should be feasible to produce enough seeds to make yam propagation through seed a reality on a commercial basis. The increase in flowering and fruiting of plants produced from seed, did not reduce tuber yield. Tubers of up to 9 kg per plant were produced by plants from seed. It was notable that non-flowering plants yielded less than flowering plants and pistillate lines yielded more than staminate lines, thus suggesting that attention should be given to the former in selecting for high yields.

In a continued effort to increase our germ plasm collection by propagating plants from seeds, mature yam fruits were collected in October and November 1973 from eight sources in Nigeria. The seeds were germinated at the termination of the dormancy period as described earlier and the established seedlings were planted directly in the field. Seedling survival in the field was about 95% (Table 2), thus establishing the feasibility of planting yam seedlings directly in the field without the need for a pre-growth period in the greenhouse. Tremendous morphological differences exist between plants with regard to their canopy structure, leaf shape and size, climbing habit, stem colour and thorniness, date of flowering and abundance of flowers.

This study clearly demonstrates the feasibility of yam seed germination on a large scale and the possibility of greatly increasing the potential for improving genetical diversity and breeding resources. Whereas breeders have hitherto been limited in their selection schemes to the narrow genetic base in cultivars that have been under continuous vegetative propagation, it is now possible to select for desirable characters from almost unlimited numbers of new lines.

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Spawning pheromone in crown-of-thorns starfish

LARGE aggregations of the crown-of-thorns starfish *Acanthaster planci* L have destroyed a high proportion of the coral on certain Indo-Pacific reefs. We report that gamete release by one *A. planci* induces other ripe starfish to spawn; similar behaviour has been observed in certain other echinoderms^{1,2}. During natural spawning a pheromone is released from the gonad, which synchronises spawning in neighbouring animals and also induces starfish movement towards the spawning individual. Using a Y maze to study chemoattraction in spawning starfish, we have attempted to isolate and characterise the active gonad component.

Several starfish (between 8 and 15) were maintained in a large Y-shaped tank which allowed an equal water flow through each of the two arms towards the base. *A. planci* normally avoids light and during the day remains in a covered area at the base, only moving into the uncovered arms at night. On six occasions, however, a single starfish became active during the mid-afternoon, and subsequently spawned, after which the remaining starfish emerged and several spawned (15 animals in 34 trials). Out of the 34 trials, 29 starfish moved towards the arm containing the spawning animal and five towards the control arm ($\chi^2=15.4; P<0.001$).

When a starfish spawns in an aquarium, other starfish in the same tank or in tanks on the same filtration circuit, also frequently spawn; those that do not may, however, show behaviour typical of spawning. Spawning behaviour may also occur in response to 1-methyl adenine injected at too low a dose to cause spawning itself (C. Huxley, personal communication). This 'spawning behaviour' consists of rapid movement with all tube feet active, and with the starfish partially inverted at the water surface; it probably corresponds to the climbing behaviour observed in spawning *A. planci* in the wild^{3,4}. Similar behaviour has been observed in *Crossaster* when spawning in aquaria⁵. Daytime movement and spawning behaviour are thus more consistent responses to a spawning stimulus than actual spawning, presumably related to the physiological limitation on frequent spawning.

Daytime movement could be induced by feeding stimuli such as coral extract; thus, although induction of movement provided

Table 1 Response of *A. planci* to chemical stimuli

Substance added	% Movement	% Spawning behaviour	% Spawning	No. of trials
None	11	1	1	206
Whole gonad	77	22.5	13	31
Sperm-free solution	100	100	0	2
Sperm	87	73	0	15
Retentate	30	15	0	6
Boiled fraction	40	10	10	10
Zeocarb (pH 7)	27	4	0	26
Zeocarb NH ₃ fraction	51	13	13	15
GSS	0	0	0	6
Glycine 10 ⁻⁶ M	0	0	0	7

Observations were made during the afternoon every 15–30 min for 1 h before addition of material to the tanks and 3–7 h thereafter. Forty starfish were available for assays, two to three to a tank (50 × 60 × 25 cm). Movement was recorded if an individual starfish moved during three successive observations; 'spawning behaviour' required a single observation during the experiment. The number of trials is the number of starfish assayed with each type of material. Figures indicate the percentage of starfish responding. Whole gonad extracts were prepared by distilled water lysis of male or female gonads treated previously with GSS. Sperm solutions were obtained from tanks in which starfish had spawned spontaneously. Retentate, material remaining in a dialysis bag after dialysis, against 3 × 2 l of distilled water, of filtered whole gonad extract (usually 200 ml). Boiled fractions are gonad preparations or sperm solutions boiled for 40 min. Dialysate of whole gonad extract was passed through a 10-ml column of Zeocarb 225 cation exchange resin at pH 7. The material which failed to attach to the column is 'Zeocarb pH 7', and that which was eluted with 1 M NH₃ is 'Zeocarb NH₃ fraction'. The gonad extracts were assayed at a concentration of the total gonad from 1 starfish per 50 l.

a convenient assay, a putative spawning factor cannot be judged on this criterion alone. The active component seems to be present in the gonad (Table 1) but not firmly attached to the gametes themselves; it is dialysable and heat-stable. Kanatani⁶ reported that neither gamete-shedding substance (GSS) from radial nerves nor 1-methyl adenine act as pheromones in *Asterias amurensis*, although both are intimately involved in the internal control of spawning. We confirm that GSS has no effect when added to the aquarium, but the starfish could detect 1-methyl adenine at 10⁻⁸–10⁻⁹ M when applied externally. All eight starfish tested extended their previously retracted tube feet, but only one starfish showed full spawning behaviour. No response was obtained, however, at concentrations up to 10⁻⁷ M, although the spawning season had ended by this time. The role of 1-methyl adenine has yet to be confirmed.

Ovary and testis extracts (data not shown) are equally rich sources of active spawning factor and in contrast to certain echinoids¹, there seems to be no differential sensitivity between males and females. We have no evidence for a strict periodicity in the responsiveness to extracts; however, those animals which had spawned recently or tended to spawn most frequently in the aquarium were more responsive than those which spawned rarely or not at all.

Whereas gonad development seems to be largely controlled by seasonal factors, the triggering stimulus for spawning in an aggregated population may be the release of gametes by neighbouring starfish. Spawning can occur in isolated individuals (D.H.B., N.J.H., and R.F.G.O., unpublished) but probably results in negligible fertilisation. In the case of mini-aggregations⁷, however, in which up to 15 starfish may be clustered around a single coral head (such as a large table of *Acropora*), a pheromone capable of attracting starfish over a distance of a few metres and also synchronising gamete release, would greatly increase the success of spawning. It has already been suggested⁸ that a large increase in spawning efficiency caused by aggregation formation may contribute to the instability of *A. planci* numbers and the occurrence of periodic population explosions.

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Production of verbenol pheromone by a bacterium isolated from bark beetles

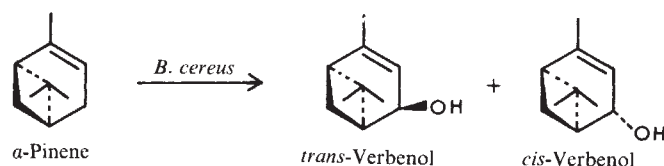
THE aggregation pheromones of the bark beetle, *Ips paraconfusus* Lanier¹ are expelled in the faecal pellets of male beetles feeding on the phloem of *Pinus ponderosa*^{2,3}. These substances, *cis*-verbenol, ipsenol, and ipsdienol⁴, seem to originate in the hindgut⁵ but the precise site of their biosynthesis has not been determined. In various species of *Ips* a connection has been convincingly demonstrated between the production of these pheromones in the hindgut and either ingestion of phloem or exposure of the beetles to host plant oleoresin⁶. More specifically, exposure of individuals of certain species of *Ips* to myrcene can result in an increased production of ipsenol and ipsdienol⁷ and exposure of certain species of *Dendroctonus* to α -pinene results in an increased production of *cis*- and *trans*-verbenol^{8–10} in the hindgut. It seems therefore that a precursor-product relationship exists between certain host plant substances such as α -pinene and myrcene and the three aggregation pheromones mentioned above.

A symbiotic relationship between an insect and a species of bacterium with respect to the production of the insect's sex pheromone, has been found in the New Zealand grass grub beetle¹¹ which carries a phenol-producing bacterium in the colleterial glands of the female. To our knowledge, however, the presence of microorganisms within the gut of an insect capable of transforming certain precursors to its pheromones has not been suggested.

Certain microorganisms can transform α -pinene into various substances including *cis*-verbenol and verbenone^{12–15}. We therefore isolated a number of different microorganisms from the gut of adult male and female *I. paraconfusus* and determined their ability to transform α -pinene into *cis*- and *trans*-verbenol. All isolated species were grown aerobically in 100 ml of 3% yeast extract in a mineral salts medium¹⁶ and shaken continuously. Good growth occurred in all cases within 24 h at which time 0.5 ml α -pinene was added and the flasks shaken for a further 24 h. Controls without either the microorganisms or α -pinene were run in the same manner. All flasks were extracted with ether, and, based on retention data of temperature-programmed gas chromatographic runs of the extracts (10% SP-1000, 60 °C to 200 °C at 10 °C min⁻¹), one organism in particular seemed to be producing the verbenols and this was identified as *Bacillus cereus*. This was isolated from both male and female beetles. Additional cultures of this organism were grown in 10 ml of the same medium, shaken for 24 h, and 0.1 ml α -pinene, purified by preparative gas chromatography, added for a further 24 h. Ether extracts of the cultures, and of

appropriate controls, were prepared and an aliquot of each extract analysed by gas chromatography on 10% EGS (140 °C). Peaks corresponding to both *cis*- and *trans*-verbenol were found in the culture extracts while none was detectable, even at high sensitivity, in the controls.

The verbenols were purified from the remaining culture extracts by the consecutive separation, collection, and rechromatography of the appropriate peaks on four different stationary phases. These were, in order of use, 10% Apiezon L (140 °C), 10% SP-1000 (140 °C), 10% EGS (140 °C), and 3% OV-225 (100 °C). Mass spectra of the purified substances eluting from the OV-225 column, at the same retention time as *cis*- and *trans*-verbenol, were recorded and the congruence of these spectra with those of standard spectra, also purified by gas chromatography, confirmed that both verbenol isomers had been synthesised from α -pinene by *B. cereus*. The yields of *cis*- and *trans*-verbenol were approximately 0.1% and 1% respectively of the added α -pinene. Another major product of this transformation has been tentatively identified as myrtenol.



In spite of the numerous studies on the aggregation pheromones of *Ips*, the site of their synthesis has not been clearly defined. Hughes¹⁰ has argued that detection of the verbenols and other oxidation products in the haemolymph of the bark beetles, *D. ponderosae* and *D. valens*, after exposure to α - or β -pinene, established that their production occurs outside the alimentary canal. It is assumed that these oxidation products are secreted into the hindgut and concentrated there by the reabsorption of water. In contrast to this hypothesis, we consider that exposure of an individual beetle to the vapour of a particular monoterpene will saturate both the haemolymph and the gut contents with the monoterpene. The oxidation of α - or β -pinene by microorganisms within the gut would then account for the greatly increased amounts of their oxidation products in this region and the detectable amounts in the haemolymph could occur either by diffusion out of the gut or by production of small amounts in the haemolymph, or by both processes.

We have found that α -pinene, the most likely precursor of both the verbenols and myrtenol, is a major component of the monoterpenes present in the phloem of ponderosa pine. Therefore, by either feeding or exposure, or both, this monoterpene would be present in the gut of male *I. paraconfusus* during nuptial chamber excavation. The occurrence of α -pinene throughout the gut of feeding *I. calligraphus*⁶ has been shown, and a number of *Dendroctonus* species produce increased amounts of the verbenols in their hindgut after exposure of individuals to α -pinene⁸⁻¹⁰. These and other observations indicate that either ingestion of α -pinene, or intimate contact with α -pinene, or a combination of both of these conditions, is a prerequisite for the production of the verbenols by the various bark beetles. It is obvious that under natural conditions of a beetle excavating a gallery both mechanisms would be operative. *B. cereus* present in the gut must come into contact with this monoterpene and presumably could bring about the oxidation of α -pinene to the verbenols. Significantly, myrtenol is also known to be present in the gut of certain bark beetles after exposure to α -pinene^{8,10}.

A *Bacillus* sp. capable of converting α -pinene to the verbenols has also been isolated from both males and females of *I. grandicollis* and three species of *Dendroctonus*. While our preliminary results do not prove conclusively that *B. cereus* actually synthesises the verbenols from α -pinene in the hindgut, our data clearly indicate that this is a distinct possibility and substantiate the hypothesis that microorganisms may play a significant role in the synthesis of certain pheromones occurring

in the frass of these bark beetles. The known differences in the quantitative production of the aggregation pheromones between males and females in these two genera remains enigmatic and is the subject of continuing investigations.

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Stability of feasible predator-prey systems

ROBERTS¹ added a new dimension to the stability analysis of model ecosystems—feasibility, the requirement that equilibrium densities of species be positive. Previous work²⁻⁴ studied the stability of randomly constructed interaction matrices, *S*, which represented ecological interactions in the neighbourhood of an assumed feasible equilibrium point.

Investigating the normal sort of quasi-linear ecological model, from which both equilibrium densities and stability can be determined, Roberts concluded that "almost all feasible systems are stable when the number of interacting species increases without limit"¹. Since real ecosystems are, *ipso facto*, feasible, this implies large ecosystems are more stable than small ecosystems—the tropics are more stable than the arctics. This is the first instance where it is claimed that mathematics has supported the conventional wisdom of the natural historians: diversity begets stability. Mathematical analysis, summarised by May⁴, has controverted this, yielding a new conventional wisdom: stability permits diversity. Roberts used the model,

$$dN_i/dt = N_i(r_i - \sum_j a_{ij}N_j), \quad i=1 \text{ to } m, \quad (1)$$

in which he assumed $r_i = 1$, $a_{ii} = -1$ and $a_{ij} = \pm z$ where $+$ and $-$ are equally likely. I have investigated the feasibility and stability of this model as a function of the interaction strength, z , and the number of species, m (Table 1). I have done the same for a modified version of this model in which $r_i = \pm 1$ and $a_{ii} = \pm 1$ where $+$ and $-$ are equally likely, and where a_{ij} are as before (Table 2).

Table 1 Feasible cases in 500 randomly constructed instances of Roberts' model. The stable cases are indicated in the parentheses

	Strength of interaction (z)					
	0.2	0.4	0.8	1.6	3.2	6.4
No. of species (m)						
2	500 (500)	500 (500)	500 (500)	133(0)	129 (0)	125 (0)
3	500 (500)	395 (395)	411 (411)	95 (20)	86 (12)	101 (16)
4	500 (500)	305 (305)	260 (257)	51 (3)	35 (5)	21 (4)
5	480 (480)	193 (191)	136 (127)	24 (3)	28 (5)	19 (3)
6	446 (446)	116 (114)	77 (63)	8 (1)	9 (1)	12 (2)
7	389 (389)	58 (53)	34 (32)	3 (0)	3 (0)	3 (1)
8	328 (328)	31 (29)	17 (11)	1 (0)	4 (2)	2 (1)
9	262 (262)	11 (11)	4 (3)	0 (0)	4 (0)	1 (1)
10	228 (228)	7 (5)	2 (2)	0 (0)	1 (1)	1 (0)

For both models, feasibility decreases with increasing m . Non-feasibility corresponds reasonably to the competitive exclusion of one or more species. (It can mathematically, but unreasonably, correspond to ecosystems whose biomass grows exponentially without limit.) Therefore, both models predict greater instability, in the sense of increased frequency of competitive exclusion, in randomly constructed systems with greater numbers of species. This is certainly no support for the conventional wisdom of the natural historians.

The two tables indicate, moreover, that the stable percentage of the feasible cases behaves differently for the two models, increasing with m in Roberts' model, decreasing with m in the second model. This means, at least, that Roberts' conclusion is fragile—not robustly insensitive to his assumptions. And it suggests that his results may be artefactual.

This is certainly the case. Because he assumed r_i to be positive for all values of i , each of his species is autotrophic; that is, each can grow in the absence of all other species. Alternatively, the species are heterotrophs and their energy supplies are implicitly included. Therefore there is neither predation nor resource competition in his model, but only

interference and facilitation (each equally likely) between species on a trophic level. Mutual interference is reasonable⁵, but the other interactions between pairs are evolutionarily absurd. Why should the members of one species facilitate the members of a species that are interfering against them? Furthermore, mutual facilitation does not commonly occur between species on the same trophic level (interspecific bird flocks may be a counter example). Odum⁶ states, "mutualism is most likely to occur between organisms of widely different requirements".

The second model does permit multiple trophic levels and predator-prey interactions. Even though it permits some absurdities (1 eats 2, who eats 3, who eats 1), it is clearly a more reasonable model of ecological interactions than the model Roberts chose. The conclusions from the second model are consistent with the mathematical analysis of diversity and stability summarised by May⁴.

One apparent difference exists between the second model and May's work: stability increases with z in the second model ($P < 0.01$), whereas it decreases with interaction strength in May's work. The interaction matrix, a_{ij} , of the second model

Table 2 Feasible cases in 500 randomly constructed instances of the second model. The stable cases are indicated in the parentheses

	Strength of interaction (z)						Average percentage stable
	0.2	0.4	0.8	1.6	3.2	6.4	
No. of species (m)							
2	119 (22)	122 (29)	120 (28)	117 (39)	109 (39)	132 (43)	28.3 ± 3.0
3	67 (8)	57 (8)	49 (5)	62 (14)	69 (17)	61 (19)	19.1 ± 3.3
4	30 (3)	35 (0)	33 (3)	29 (7)	33 (7)	29 (4)	13.0 ± 3.6
5	17 (1)	20 (4)	17 (1)	15 (3)	22 (4)	10 (1)	13.0 ± 2.9
6	5 (0)	7 (1)	12 (0)	7 (0)	11 (0)	3 (0)	2.38 ± 2.3
7	3 (0)	5 (0)	8 (2)	5 (0)	3 (0)	4 (2)	12.5 ± 8.5
8	1 (0)	4 (0)	1 (1)	3 (1)	1 (0)	1 (0)	19.0 ± 14.2
9	2 (0)	3 (0)	2 (0)	1 (0)	4 (0)	1 (0)	0
10	0 (0)	1 (0)	0 (0)	0 (0)	1 (0)	1 (0)	0
Total stable cases	34	42	40	67	67	69	

is not, however, the stability matrix. This is $S_{ij} = \bar{N}_i a_{ij}$, where $\bar{N}_i$ is the equilibrium density of species i ($\bar{N}_i = \sum_j a_{ij}^{-1} r_j$). For the second model, the average and median $\bar{N}_i$ decrease with z . Biologically, the predators exploit the prey beyond the optimal yield, lowering the densities of all populations. This is certainly the basis for the above noted discrepancy. A deeper investigation into this phenomenon is in progress.

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Influence of menstrual cycle on volunteering behaviour

RANDOM population sampling is rarely achieved in human research. Rosenthal and Rosnow¹ have concluded that human behavioural science is largely the science of punctual college sophomore volunteers. Volunteers typically have more education, higher occupational status, earlier birth position, lower chronological age, higher need for approval and lower authoritarianism than nonvolunteers²⁻⁹. We report evidence here to suggest that the volunteering behaviour of college women varies with the stage of their menstrual cycle. This implies that many experiments which report perceptual, cognitive or physiological response differences between the sexes may be biased in systematic fashion by sampling error.

In the initial experiment, women from two sections of a University of San Francisco child psychology class completed, in class, a questionnaire consisting of eight items on attitudes towards family planning, alcohol and drug use on campus, and peer-group and parental relationships. Each respondent's age, social security number, religion, average menstrual cycle length, use of oral contraceptives and date of onset of the most recent *menses* were also obtained. Social security numbers were used for data tabulation and the respondents were assured that the information would be kept confidential and used only for scientific purposes. Two days later a male social psychologist, not associated with the original questionnaire, was introduced to the class and briefly described a research project for which he needed volunteers. For one section, the proposed project required that the volunteers work alone on a learning task. For the other it required that they work as a group in a decision-making task. Sign-up sheets were subsequently passed around the class. As in many psychology classes, such requests for volunteers were a common occurrence.

To establish whether the stage of the menstrual cycle was related to volunteering, the cycle of each woman not taking oral contraceptives was divided into five phases on the basis of the questionnaire data. The midpoint of the 'ovulatory phase' was calculated from the reported average cycle length by subtracting 14 d. The 2 d on each side of this midpoint

were included in this phase, making it 5 d long. The 'menstrual phase' was calculated as 5 d extending from the onset of menstrual bleeding; the 'preovulatory phase' made up the days falling between the menstrual and ovulatory phases, whereas the 'postovulatory phase' made up the first half of the period extending from the end of the ovulatory phase to the beginning of the next presumed *menses*. The second half of this period was termed the 'premenstrual phase'.

Since the type of experiment did not affect the frequency of volunteering, the data for both lecture sections were combined and are presented in Table 1a. Most of the volunteers were in the ovulatory phase, whereas most of the nonvolunteers were in the postovulatory, premenstrual and menstrual phases ($\chi^2 = 22.47$, d.f. = 4, $P < 0.001$).

To establish whether this phenomenon was dependent on the sex of the individual seeking volunteers, we repeated the experiment a year later in a different class section using a female social psychologist. In this case, the questionnaire data were obtained on the same day as the request for volunteers. Table 1b shows that the results were essentially the same as in the previous experiment ($\chi^2 = 25.31$, d.f. = 4, $P < 0.001$).

Direct evidence for this phenomenon is difficult to find in the literature, since few investigators report the testing order of volunteers within the menstrual cycle. In a recent study of taste preferences, Wright and Crow¹⁰ divided the menstrual cycle of their volunteer subjects into five equal phases. It is interesting that the number of subjects tested in the five phases (sequentially from *menses*) was 9, 18, 24, 25 and 17, respectively ($\chi^2 = 8.88$, d.f. = 4, $0.05 < P < 0.10$). Many behavioural studies reporting sex differences have assumed a random distribution of volunteering during the menstrual cycle because no relevant data were collected, and even studies examining menstrual cycle phenomena directly have made such an assumption. For example, the authors of one study¹¹ reporting changes in visual sensitivity during the menstrual cycle stated: "Because they were initiated into the test program as they volunteered, session order for the females was independent of the phase of the menstrual cycle". Our results suggest that this assumption is questionable and that test order should be counterbalanced so that systematic order effects are not confounded with endocrine influences.

Several explanations of our findings seem plausible. Some women, at least in Western cultures, undergo cyclic changes in emotions during the menstrual cycle. Premenstrual and menstrual increases in tension, irritability, depression, anxiety and fatigue have been reported, as have increases in elation and activity near the time of ovulation¹²⁻¹⁴. Such psychological and physiological changes may influence directly the tendency of women to volunteer for research. It is possible that tendencies to respond to convert or overt classroom teacher and/or peer-group pressure are related to changes in 'response criteria' noted in signal detection analyses of perceptual phenomena during the menstrual cycle¹⁵.

Regardless of the specific causal factors involved, our results suggest that many experiments which use female volunteers test a disproportionate number whose hormonal status is skewed from that of the general population of females. The degree of skewing would depend on factors such as the length of time between the act of volunteering and actual participation in the experiment. If such participation has followed soon after the time of volunteering, much of the literature on human females may be representative of punctual college sophomore

Table 1 Number of volunteers and nonvolunteers in each of the five phases of the menstrual cycle

Phase	Menstrual	Preovulatory	Ovulatory	Postovulatory	Premenstrual
a Male experimenter					
Volunteers	0	7	14	5	3
Nonvolunteers	7	4	1	12	5
b Female experimenter					
Volunteers	3	2	7	1	0
Nonvolunteers	23	4	1	14	4

volunteers who are probably not menstruating and are of ovulatory status.

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Differential rates of cerebral maturation between sexes

TAYLOR and Ounsted^{1,2} have proposed a model to account for the age of onset of developmental disorders and their relative incidence between the two sexes. They propose that there is a period in the maturational continuum during which the child is particularly vulnerable to a particular disorder. Males develop more slowly than females and they should therefore reach this "dangerous state" later, and take longer to pass through it. It is therefore predicted for any developmental disorder, that males should show a later age of onset and a higher incidence than females²; but that females are more likely to suffer seriously than males¹, (although this does not follow directly from the model). Evidence relevant to these predictions is reported here for congenital hydrocephalus.

Medical records are available for 48 children who were referred to the Neurosurgical Unit at the Radcliffe Infirmary, Oxford, over the past 20 years, and who were diagnosed as suffering from congenital hydrocephalus. In most cases there was no associated spina bifida cystica or other complex multiple congenital deformities. For each patient, it was established whether surgical intervention had been necessary, or whether the hydrocephalus had arrested spontaneously (Table 1).

The series contained 33 males and 15 females. This is equivalent to a sex ratio of 220 males for every 100 females, and differs significantly from equiprobability according to the binomial test ($z = 2.56$, $P < 0.01$, two-tailed test). The median age at which the boys were referred was 26 weeks; the median age at which the girls were referred was 7 weeks. The Mann-Whitney U test demonstrated that the boys were significantly older when referred than the girls ($z = 1.69$, $P < 0.05$, one-tailed test).

In the case of 23 of the children (48%), the hydrocephalus

arrested spontaneously. This compares closely with a figure of 46% reported by Laurence and Coates³ from a much larger series. Considering boys and girls separately, it was found that spontaneous arrest occurred in 17 (52%) of the boys, and in 6 (40%) of the girls. Thus, the girls were more likely to require surgical intervention than the boys. The difference is not statistically significant, however, according to a χ^2 test which used the correction for continuity ($\chi^2 = 0.18$, d.f. = 1, $P > 0.5$). The median age at which a patient was referred if his hydrocephalus arrested spontaneously was 48 weeks; the median age at which he was referred if surgery was necessary was 17 weeks. The difference between these two medians approaches statistical significance, according to a Mann-Whitney U test ($z = 1.82$, $P < 0.1$, two-tailed test). Both groups, however, showed the effect of sex on the age at which they were referred, described above.

These results support the two principal hypotheses which can be derived from the model proposed by Taylor and Ounsted². Congenital hydrocephalus occurs in boys later and more often than it does in girls. This constitutes further evidence for two generalisations in the epidemiology of disease: females are susceptible earlier than males; yet males are more susceptible than females. The model proposed by Taylor and Ounsted links these two generalisations by a single formal mechanism.

If the necessity for surgical intervention can be taken as a sign of the severity of hydrocephalus, there is also some evidence for their assertion that diseases tend to occur more severely in females than in males. This evidence is, however, not supported by statistics. Nevertheless, the findings of this study suggest that research into developmental disorders may increase our understanding of the nature of sex differences and the processes of cerebral maturation.

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Nude mice with normal thymus

MICE homozygous for the mutation, nude (*nu*) have been reported to have no thymus¹, although they retain a pre-lymphoid thymic rudiment². Here, I report the occurrence of individuals that are by external signs (including hairlessness) a nude mutation but possess an apparently normal thymus.

The outbred nude stock in this laboratory has been repeatedly reinforced with $+/nu$ heterozygotes and some fertile nu/nu homozygotes (Laboratory Animals Centre, Carshalton). The ensuing appearance of 'nudes with thymus' suggested that this phenotype originated from Carshalton, but the alternative could not be excluded that either a new mutation or some infiltration of a hairless gene might have happened in this laboratory. Similar findings were communicated to me by Dr D. Wakelin of the Wellcome Laboratories for Experimental Parasitology at the University of Glasgow, who had obtained his breeding stock from me. A Carshalton origin, however, seemed to be confirmed when in November, 1974, I obtained from there a large group of nude mice aged about 3 months. Out of over 60 autopsied so far there was one with an apparently normal thymus and one with a minute 'thymus'. These tissues are being examined.

Table 1 Number of patients referred, by sex and age at referral

	Age (months)							
	0	1	2	3-5	6-8	9-11	12-23	24 and over
Males	1	5	3	7	5	3	5	4
Females	5	4	0	0	1	1	2	2

In an attempt to test the hypothesis that a crossing over might have occurred between the gene for athymia and a separate, so far unsuspected, gene for hairlessness, 48 young were obtained from matings of animals supplied by Dr Wakelin and known to have given previously at least one nude mouse with a thymus. Although such phenotypes again occurred, not a single animal was found with a normal coat but no thymus. Several animals with unusually small thymuses were observed, but the significance of this is problematical.

Work is continuing on the above and alternative hypotheses, such as selection of modifiers under systems of husbandry where, in outbred stocks, nudes that happen to be fertile are favoured as breeders³. The present interim report becomes necessary because of the hazard the occasional nude mouse with a normal thymus constitutes for the experimenter. It is now advisable to ensure that all nude mice are tested for athymia at autopsy.

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Nonspecific and specific immuno-suppression in tumour-bearing mice by soluble immune complexes

SEVERAL reports have concerned themselves with specific suppression (blocking) of tumour-immune responses by serum factors from tumour-bearing animals^{1–3}, and nonspecific depression of T lymphocyte responses in tumour-bearing mice has also been described^{4,5}. Evidence exists that specific blocking of lymphocyte-mediated immunity occurs by means of either free antigen⁶ or antigen-antibody complexes⁷. It has now been suggested that nonspecific suppression of lymphocyte responses could also occur by means of immune complexes⁸, if one assumes that both T and B lymphocytes have Fc receptors for the antibody region of such complexes. There is a growing body of evidence for Fc receptors of T cells^{9,10}. We have investigated the possibility that the nonspecific depression of the T lymphocyte phytohaemagglutinin (PHA) response seen in BALB/c mice carrying tumours, induced by Moloney sarcoma virus (MSV) in the progressive phase of growth⁴, is caused by similar factors to those which cause specific blocking in this system. Our results suggest that while tumour-specific blocking can be mediated by free antigen, as well as antigen-antibody complexes, nonspecific blocking of T-cell responses is mediated only by the latter.

The technique for stimulation of DNA synthesis as an assay for T lymphocytes responding to PHA or MSV tumour antigen is reported elsewhere⁴. A ⁵¹Cr assay which measures specific cytotoxic T lymphocytes was also used, the targets being derived from MSV-infected mouse fibroblasts passaged once through young BALB/c mice¹¹. All cultures were performed in Dulbecco's modified Eagles MEM, supplemented with 10% foetal calf serum (DF₁₀). Cells capable of nonspecific suppression of immune responses were obtained from spleen cells from MSV regressor animals by velocity sedimentation⁴. Such cells represent that fraction of the total spleen population which sediments with velocity 4.8–6.0 mm h⁻¹. Mouse sera were obtained by cardiac puncture, the serum being heat-inactivated (56 °C for 30 min) and stored at –20 °C until use. Antigen-antibody complexes were eluted from the kidneys of normal and MSV-infected mice by the method of Oldstone and

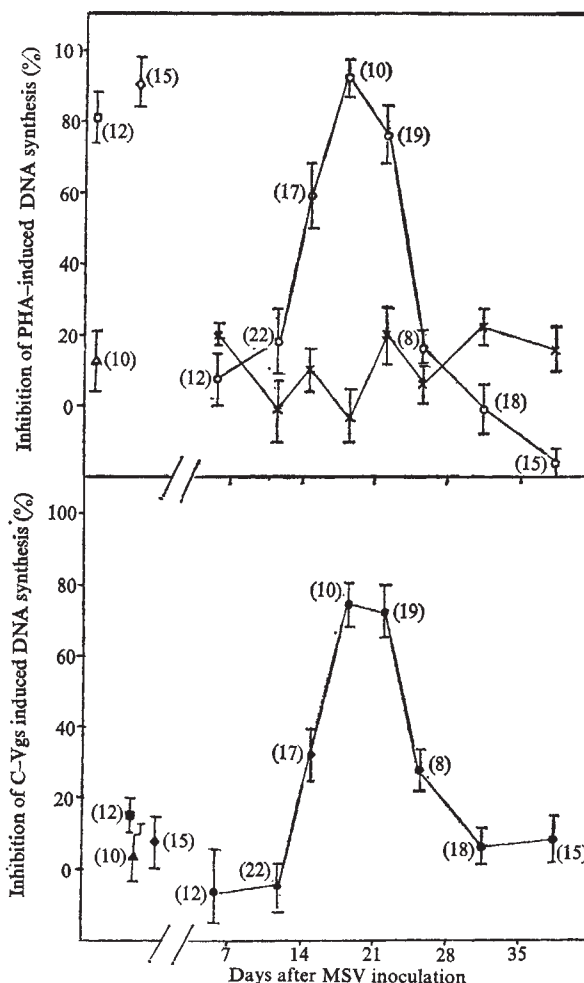


Fig. 1 Effect of tumour bearer and tumour regressor sera on specific and nonspecific T-cell responses. The assay used to test for the suppression of the T lymphocyte response was to compare the stimulation index

$\left(\frac{\text{c.p.m. } ^3\text{H-thymidine incorporated in the presence of stimulus}}{\text{c.p.m. } ^3\text{H-thymidine incorporated in the absence of stimulus}} \right)$ of 4×10^6 MSV regressor spleen cells (from mice given MSV 30 d before) incubated in DF₁₀ in the presence or absence of the various sera. For suppression of PHA responses (open symbols) and LPS responses (crosses) the sera were used at a 1:10 final concentration; for suppression of C-Vgs responses (closed symbols) the sera were used at a 1:50 final concentration. The sera tested were: ○, ●, ×, MSV inoculated animals; △, ▲, normal mice; □, ■, polyoma tumour bearers; ◇, ◆, fibrosarcoma tumour bearers. The number of mice used to provide the pool of serum tested is shown in brackets beside the relevant symbol. All groups were set up in triplicate. 95% confidence limits are shown for each point. The stimulation indices of the MSV regressor cells used in the presence of DF₁₀ alone were: PHA, 19 ± 2.3 ; LPS, 23 ± 4.1 ; C-Vgs, 6.2 ± 1.2 .

Dixon¹³. Rabbit anti-mouse immunoglobulin (anti-MIg) columns were prepared as described previously¹⁴. Absorption of mouse sera on these columns (maximum capacity 2 mg immunoglobulin) was performed at pH 7.0 in phosphate buffered saline (PBS). Serum (0.5 ml) was washed on to the column with 1 ml of PBS, the column left at room temperature for 30 min, and the column was then washed with PBS until the absorbance at 280 nm was less than 0.05. The absorbed immunoglobulin was eluted at pH 2.6 with HCl glycine buffer. The pH of the eluted immunoglobulin was adjusted to 7.0 with phosphate buffer and both the unabsorbed effluent and the eluted immunoglobulin were concentrated by vacuum dialysis and dialysed against 200 volumes of PBS for 24 h.

Figure 1 indicates the ability of serum taken from animals at different times after MSV infection to inhibit the induction of DNA synthesis by PHA, lipopolysaccharide (LPS), or MSV

Table 1 Effect of MSV sera or suppressor cells on response to PHA and C-Vgs

Velocity-sedimented suppressor cells added per culture	Final concentration of MSV serum present per culture	Stimulation index in response to	
		C-Vgs	PHA
None	None	5.5 ± 1.1	24 ± 3.6
2 × 10 ⁶		0.9 ± 0.2	4.3 ± 2.1
1 × 10 ⁶		1.1 ± 0.3	6.2 ± 2.1
0.2 × 10 ⁶		1.8 ± 0.4	18 ± 4.6
0.1 × 10 ⁶		3.2 ± 0.5	19 ± 3.7
	20%	0.8 ± 0.3	2.6 ± 1.3
	14%	0.9 ± 0.2	6.3 ± 2.1
	6%	1.2 ± 0.2	10.5 ± 3.2
	3%	2.9 ± 0.5	18 ± 4.1

2 × 10⁶ spleen cells from MSV regressor animals (25 d after MSV inoculation) were cultured for 72 h in the presence of the stimulants shown (PHA, 1 µg ml⁻¹; C-Vgs, 5 µg ml⁻¹), with or without varying amounts of suppressor material. All cultures were set up in triplicate. The cultures were washed, pulsed for 12 h with 1 µCi of ³H-thymidine, and the TCA-precipitable material collected and counted in a liquid scintillation counter. The stimulation index was calculated as in the legend to Fig. 1. 95% confidence limits were calculated taking into account the variation in both numerator and denominator.

tumour-associated antigen (in this case a cell surface antigen with viral group specificity, C-Vgs). It is clear from these data that at around the time of tumour regression, serum factors appear which can block either of the T lymphocyte-mediated responses but not the B-cell response. Moreover, the blockade of the C-Vgs response was mediated by lower concentrations of serum than were needed to cause nonspecific blocking. The nonspecific blocking was seen with sera from other tumour-bearing mice (described elsewhere¹⁵), but the low concentration effect of serum on the C-Vgs induced enhanced DNA synthesis seems to be tumour specific¹¹. In our experiments normal mouse serum has never had a consistent suppressive effect on the splenic response to T or B cell mitogens (see Fig. 1 and Table 2).

The correlation between the ability of sera taken from animals at different times after MSV infection to inhibit T lymphocyte responses in both a specific and a nonspecific manner agrees well with the simultaneous fall of each type of response in the spleen of MSV progressor animals⁴. It is tempting to speculate that both the same serum factors and the same suppressor cell (already shown to be an immunoglobulin-bearing cell with sedimentation velocity 4.8–6.0 mm h⁻¹) cause these effects,

and that specific suppression is observed when the concentration of suppressor is limiting. Data to support this idea are shown in Table 1. On dilution of sera or velocity-sedimented suppressor cells, the PHA response obtained from MSV regressor animals is restored before the C-Vgs response. This is consistent with the notion that the same cell type (or molecule) produces each effect, the difference between seeing specific or nonspecific inhibition being caused by the concentration of cells (or molecules) needed to see each effect.

In order to examine the possibility that immune complexes cause both specific and nonspecific inhibition of T lymphocyte function we have used an anti-MIg column in an attempt to remove the blocking activity obtained from different sources. Three independent sources of blocking material have been used: (1) serum taken from mice 20 d after MSV inoculation (Fig. 1); (2) material eluted from the kidney basement membrane of normal and the mice 20 d after MSV inoculation, following a method previously described for obtaining immune complexes from such sources¹³; (3) supernatants from 24 h cultures of velocity-sedimented suppressor cells from MSV progressor animals, or their normal spleen cell equivalent. Each supernatant was prepared from 1 × 10⁸ cells, incubated

Table 2 Removal of blocking factors by an anti-immunoglobulin column

Source of blocking activity (concentration in PHA cultures; concentration with C-Vgs and in ⁵¹ Cr assay)	Stimulation of DNA synthesis by		% Specific ⁵¹ Cr release
	PHA	C-Vgs	
None	21 ± 3.2	6.5 ± 1.2	53 ± 2.2
Normal serum (4mg ml ⁻¹ ; 800 µg ml ⁻¹)	22 ± 2.6	6.7 ± 1.1	52 ± 1.9
Unfractionated (4 mg ml ⁻¹ ; 800 µg ml ⁻¹)	4.1 ± 1.7	0.7 ± 0.2	14 ± 2.2
20 d after MSV	16 ± 2.7	2.8 ± 0.3	40 ± 1.7
Column effluent	9.9 ± 2.1	2.1 ± 0.4	35 ± 1.9
Column absorbed, acid-eluate	6.8 ± 2.4	1.3 ± 0.4	15 ± 1.5
Recombined fractions			
Normal supernatant (400 µg ml ⁻¹ ; 150 µg ml ⁻¹)	22 ± 1.7	6.7 ± 1.3	52 ± 2.1
Unfractionated (400 µg ml ⁻¹ ; 150 µg ml ⁻¹)	7.8 ± 2.1	2.9 ± 0.3	48 ± 1.9
Suppressor cell	19 ± 2.6	5.8 ± 0.5	50 ± 2.2
supernatant	21 ± 2.1	5.3 ± 0.4	53 ± 2.3
Column effluent	17 ± 3.2	6.2 ± 0.3	51 ± 1.9
Column absorbed, acid-eluate			
Recombined fractions			
Normal kidney extract (1 mg ml ⁻¹ ; 300 µg ml ⁻¹)	22 ± 2.2	6.6 ± 0.9	55 ± 2.3
Unfractionated (1 mg ml ⁻¹ ; 300 µg ml ⁻¹)	5.1 ± 1.6	1.3 ± 0.2	17 ± 1.9
20 d after MSV	19 ± 2.1	5.8 ± 0.3	47 ± 1.7
Column effluent	18 ± 3.1	6.4 ± 0.9	51 ± 2.0
Column absorbed, acid-eluate	8.2 ± 3.3	3.1 ± 0.3	28 ± 2.2
Recombined			

Stimulation of DNA synthesis (using 4 × 10⁶ regressor cells per culture) was as described in Table 1 and elsewhere¹⁶. The final data are expressed as an arithmetic mean stimulation (compared with non-stimulated cells) with 95% confidence limits. The ⁵¹Cr cytotoxic assay (final column) is described in more detail elsewhere¹¹. Briefly, 7.5 × 10⁶ regressor cells were incubated with 5 × 10⁴ ⁵¹Cr lymphoma cells for 15 h at 37 °C; the final volume of the culture medium was 1 ml, and this medium also contained the blocking substances shown. Percentage specific ⁵¹Cr release was calculated as

$$\left(\frac{\text{c.p.m. in supernatant with immune cells} - \text{spontaneous released c.p.m.}}{\text{c.p.m. in supernatant with detergent} - \text{spontaneous released c.p.m.}} \right) \times 100$$

Normal cells routinely gave less than 0.5% specific release. All cultures were set up in triplicate; the concentration of blocking factors in the tests used is shown in brackets thus (concentration with PHA as antigen; concentration with C-Vgs or lymphoma cells as antigen). Anti-MIg column fractionated material was concentrated to the equivalent volume of the unfractionated materials.

in 5 ml serum-free Eagles' MEM with 5×10^{-3} M 2-mercaptoethanol, and concentrated 30-fold by vacuum dialysis before use.

Each source of blocking material was tested with MSV regressor cells in a DNA synthesis assay as in Table 1, and in a ^{51}Cr cytotoxicity test (with labelled MSV target cells). Four fractions of each blocking material were analysed; unabsorbed material, column effluent and acid-eluate, and the latter two recombined at a 1:1 ratio. The data for this experiment are shown in Table 2.

Serum, kidney extract or cell culture supernatant derived from normal animals had no significant suppressive action on the responses measured. Whereas each of the unfractionated blocking materials derived from MSV-infected mice caused both antigen-specific and nonspecific suppression of T-cell responses, the effluent from an anti-MIg column (which we presume contains free antigen) blocked only the antigen-specific responses (see effects of serum effluent on C-Vgs and PHA induced DNA synthesis, and in the ^{51}Cr assay). The column-absorbed, acid-eluted material, which could contain excess-free antibody and/or antigen-antibody complexes, again blocked the antigen-specific responses, and often showed significant blocking of the nonspecific T cell response. The best suppression of the antigen-specific response, however, and often the only suppression of the nonspecific response, was seen when column-effluent and column-absorbed (acid-eluted) material were recombined (giving antigen-antibody complexes, perhaps in antigen excess). This is particularly well documented for the MSV kidney extract, where only the recombined material had any suppressive properties.

We interpret these data as suggesting that optimum non-specific T cell blocking is mediated by antigen-antibody complexes, perhaps in antigen (column effluent) excess. Antigen-specific T cell blockade is produced by lower concentrations of such complexes, and, unlike nonspecific blocking, can also be mediated by free antigen. We feel that the failure to recover blocking activity from effluent, acid-eluate or recombined material from the fractionated suppressor cell supernatant was a result of the low concentration of suppressor material applied to the column in this case. These findings do not agree with an earlier report that in the MSV system in mice T cell cytotoxicity is not blockable by soluble tumour-associated antigen (TAA) or progressor serum³. The data do agree with the report that macrophage migration inhibition in this system, also a T cell-mediated response¹⁶, is inhibitable by progressor sera¹⁷. A recent report indicates that T-cell-mediated cytotoxicity in a rat Gross-virus induced lymphoma is also inhibitable by soluble TAA or progressor sera¹⁸.

It remains to be determined why T-cell cytotoxicity, which is sensitive to lower concentrations of blocking factors in serum and kidney extracts than the PHA response, is not blocked by the suppressor cell fraction or culture supernatants which block antigen-specific and PHA proliferative T-cell responses. We are investigating whether different TAA may be involved in T-cell cytotoxicity.

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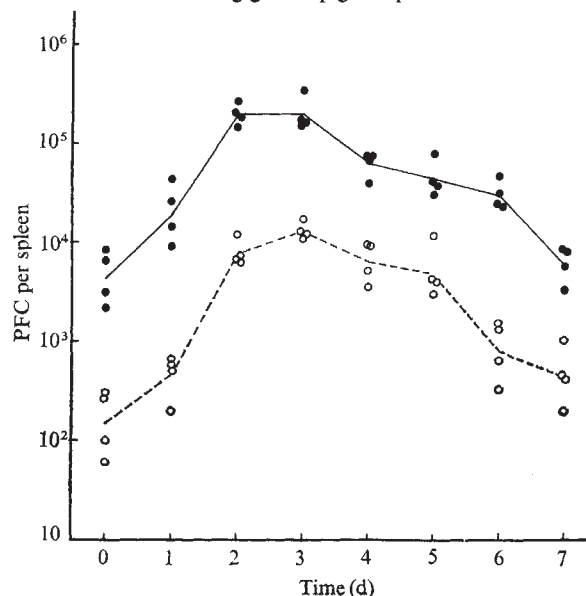
Active suppressor mechanism maintaining tolerance to some self components

NORMAL mice have in their lymphoid tissues a fairly large number of cells making antibody to antigens present within isologous erythrocytes¹. These plaque-forming cells (PFC) are readily seen on red blood cells (RBC) treated with bromelain, or other proteolytic enzymes¹. DeHeer and Edgington² have recently described similar PFC in NZB mice.

Certain facts suggest that this constant level of PFC in normal mice is maintained as a balance between two opposing forces, continual antigenic stimulation by self antigens on the one hand, and some suppressor mechanism on the other. Constant stimulation seems likely since the antigens form part of normal mouse RBC and probably become exposed as red cells degenerate. CBA mice usually have 2,000-7,000 PFC on bromelain-treated RBC by 3 weeks of age, and maintain these plaques for many months at about this level, which is some 20 times higher than the background to heterologous RBC (ref. 1). Germ-free mice have similar numbers of plaques¹. Specific suppression as a force opposing further increases in the numbers of these PFC is suggested by two observations. First, injecting bromelain-treated isologous RBC at a variety of dosage and routes gives little or no increase in PFC on similar target cells (ref. 1 and A.J.C., unpublished). Secondly, cells potentially able to make antibody or antibody-forming progeny to the internal RBC antigens are probably numerous, since mitogen (bacterial lipopolysaccharide from *Escherichia coli*) induces enormous numbers of plaques (Fig. 1).

A way of testing for suppressive effects was suggested by the experiments of Baker and colleagues^{3,4}, who showed, using antilymphocyte serum (ALS), that suppressor T cells

Fig. 1 Effect of an intraperitoneal injection of 50 μg of lipopolysaccharide on numbers of plaques to bromelain-treated isologous RBC in mouse spleen. ●, Plaques on treated mouse RBC using rabbit complement; ○, PFC against normal sheep RBC using guinea pig complement.



may diminish antibody responses to pneumococcal polysaccharide. We made ALS by injecting rabbits intravenously with $2-10 \times 10^8$ mouse thymus cells on two to four occasions separated by intervals of 15–24 d. Figure 2 shows that three injections of a total of 0.8 ml of this ALS increased the PFC numbers on bromelain-treated isologous RBC about 20-fold. This is unlikely to have been a non-specific effect (that is, one caused by some general stimulation of immunocompetent cells), for several reasons. The ALS had little or no effect on background plaques to sheep RBC (Fig. 2), in sharp contrast to the stimulation of the anti-sheep response by mitogen (Fig. 1). Normal rabbit serum had a much smaller stimulatory effect (Fig. 2). In control experiments it was shown, first, that most of the activity of the ALS could be removed by absorption with mouse thymus cells; second, that the same pool of ALS decreased a response to injected sheep RBC about tenfold; and, third, by analogy with Baker's experiments⁴, that the PFC number in nude mice was not increased by ALS injection, whereas that of their normal littermates was increased several-fold.

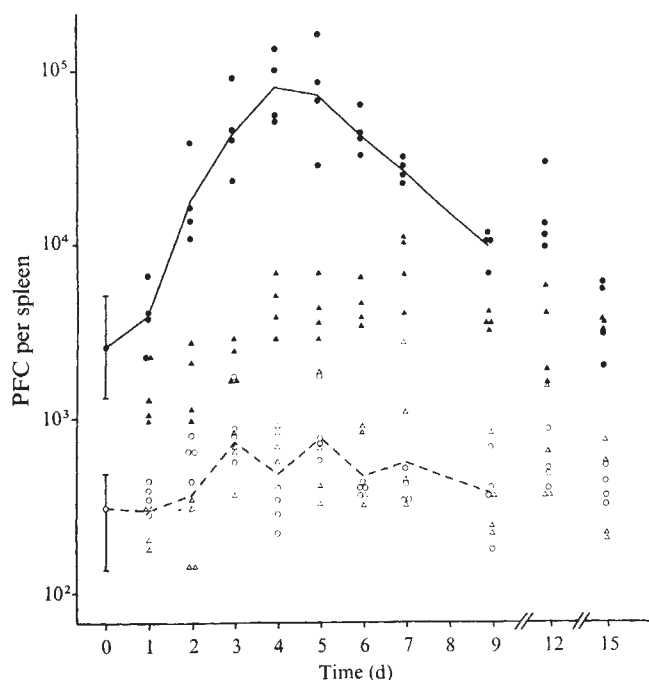


Fig. 2 CBA mice were injected with 0.2 ml ALS intraperitoneally and 0.2 ml subcutaneously on day 0. On days 2 and 4, they received 0.1 ml in each site. Control animals received serum from a normal rabbit. ●, ALS injected, PFC assayed on bromelain-treated mouse RBC. ○, ALS injected, PFC on sheep RBC. ▲, Normal rabbit serum injected, assayed on bromelain-treated mouse RBC. △, Normal rabbit serum injected, PFC on sheep RBC. The day 0 points show geometric means and range of values in 12 uninjected mice tested on three different days.

The simplest explanation for these results is that ALS promotes the autoantibody response by removing suppressor cells. The suppressors may be T cells, although the evidence is not conclusive. This example of suppressor tolerance to a group of self antigens in normal mice has obvious similarities to the active suppression, in young NZB mice, of responses to their own red cells^{5,6}.

It is important to determine the scope of this kind of mechanism by investigating a number of different model systems, since if tolerance to many self components, as well as to many foreign antigens⁷, is maintained by active suppression of potentially reactive cells, a completely new view of the workings of the immune system would emerge. In extreme form, this may be stated as follows. All antigen-reactive cells bind some self components to varying extents, and all are subject

to some degree of continuous suppression (this is not to deny that deletion of some clones may occur if they are faced with overwhelming amounts of a soluble self antigen which they bind avidly). The success of a foreign antigen in provoking a specific immune response depends on its ability to disturb this balanced state, with antigens close to self being most liable to suppression, and therefore least immunogenic. During any immune response, cells making entirely new antibody specificities are rapidly generated^{8,9}, and these new variants are also liable to suppression, so that only those which most successfully avoid the suppression mechanism are seen. Constant stimulation with a foreign antigen leads to its being treated like self¹⁰; more, and perhaps new kinds of, suppressor cells develop, and a state of greatly reduced responsiveness to that antigen eventually supervenes.

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Separation of cells involved in phytohaemagglutinin-induced mitogenesis and cytotoxicity

LYMPHOCYTE mitogens such as phytohaemagglutinin (PHA) may induce lymphocytes to mitogenesis (DNA synthesis) and cytotoxicity (lysis of target cells). Although there is evidence that these two responses are independent of each other it remains to be determined whether different lymphocyte subpopulations are involved^{1,2}. It has been claimed that PHA is a selective T-cell mitogen but some recent work argues against such precise specificity³. There is also evidence suggesting that PHA may induce T cells to become cytotoxic but, since non-T cells may be cytotoxic under some conditions, the situation remains to be clarified^{1,2,4-8}.

In the course of investigation of the lymphocyte function of patients with immunological deficiencies, we have been impressed by the frequent absence of PHA-induced mitogenesis when PHA-induced cytotoxicity seems to be normal. The reverse situation also occurs. These observations suggested that different lymphocyte subpopulations might be involved in mitogenesis and cytotoxicity. We report here evidence which shows that the cells involved may be separated by rosette sedimentation.

Between 50 and 100 ml of heparinised blood were collected from seven normal subjects and centrifuged on Isopaque-Ficol gradients⁹ with a yield of 100–200 mononuclear cells per individual. Polymorph contamination was less than 5% whereas the percentage of monocytes was variable. The separated mononuclear cells were then incubated with sheep red blood cells (SRBC) so as to allow the formation of non-immune T-cell E rosettes¹⁰. The mononuclear-SRBC mixture was then layered on to Isopaque-Ficol and centrifuged as for the initial separation. The cells collected from the interface were found to contain less than 5% E rosetting cells and from 34–61% B cells as indicated by the presence of readily demon-

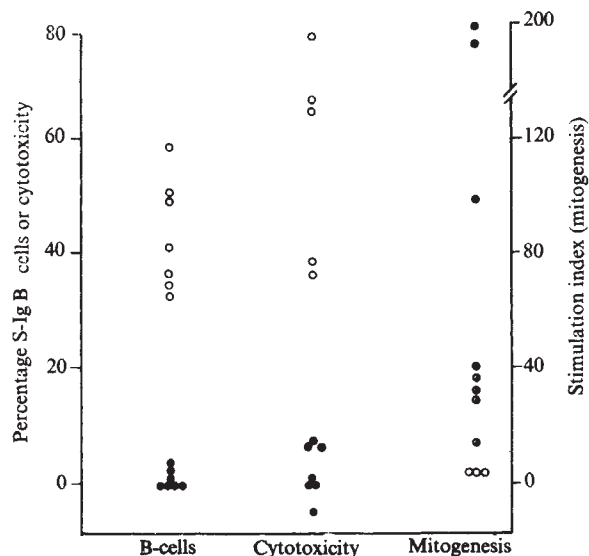


Fig. 1 The effect of rosette sedimentation on PHA-induced cytotoxicity, PHA-induced mitogenesis and B cells. ○, Cells from the interface; ●, cells from the pellet; ◐, mononuclear cells before rosette sedimentation. Stimulation index refers to counts after PHA stimulation divided by the resting level.

strable surface immunoglobulin (S-Ig)¹⁰. In contrast less than 5% and generally less than 1% of lymphocytes in the pellet were B cells with S-Ig. Thus the interface population consisted of B cells with some "null" lymphocytes (non-E-rosetting, non-S-Ig) and some monocytes but very few E-rosetting T cells whereas the pellet population consisted of essentially pure T cells.

These two populations were then compared in terms of PHA-induced mitogenesis and cytotoxicity. The former was estimated by measuring the tritiated thymidine uptake after incubating 10^6 lymphocytes with 5 μ l PHA-P (Difco) for 3 d. Cytotoxicity was determined by counting ^{51}Cr release from 10^5 labelled chicken red blood cells after 18 h incubation with 2.5×10^6 lymphocytes and 10 μ l of PHA¹¹. Contaminating SRBC were removed from the pellet by lysis with 0.83% NH_4Cl -Tris-HCl (pH 7.65) although in some experiments equivalent numbers of SRBC were added to the interface population.

Results are summarised in Fig. 1. There was a gross reduction in the PHA-induced cytotoxicity of the pellet population in relation to the interface population. The reverse situation was seen with respect to mitogenesis induced by PHA. The pellet population showed high stimulation indices relative to either the interface or the starting mononuclear population before rosette sedimentation.

These data show that different subpopulations are involved in the mitogenic and cytotoxic responses to PHA. It could be argued that the mitogenic response involves only E-rosetting T cells, but in other experiments we have found that there can be some stimulation of the interface populations and interpretation may be complicated by the fact that resting DNA synthesis is higher in the interface than the pellet population¹². For these reasons we cannot conclude that PHA mitogenesis is an absolutely specific T-cell marker but it would seem to be far more so than some others have suggested³. E-rosetting T cells seem to contribute very little, if at all to PHA-induced cytotoxicity. Whether B cells, non-E rosetting T cells, "null" lymphocytes or even monocytes are responsible for the cytotoxicity seen after incubation with PHA cannot be determined from the present study. But we believe that our data clearly indicate that PHA-induced mitogenesis and cytotoxicity should be considered as representing different functions and populations when assessing patients with immunodeficiency states.

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Arthritis and Rheumatism Foundation, Muscular Dystrophy Associations of America and the Australian Kidney Foundation. Note added in proof: Work in progress (P.J.Z., G. Grimsley and R.L.D., unpublished) suggests a major role for monocytes in PHA-induced cytotoxicity against RBC.

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Sertoli cell secretory function after hypophysectomy

TESTICULAR androgen is the principal stimulus for spermatogenesis¹. Androgens alone are capable of maintaining spermatogenesis in hypophysectomised rats when treatment is begun immediately following hypophysectomy². Following posthypophysectomy regression of the seminiferous epithelium, however, neither luteinising hormone (LH) nor androgen given alone is capable of initiating spermatogenesis when the same dose and time schedule is used for the treatment. Follicle stimulating hormone (FSH) has to be given together with LH or androgen to effectively restore spermatogenic activity¹. This difference between main-

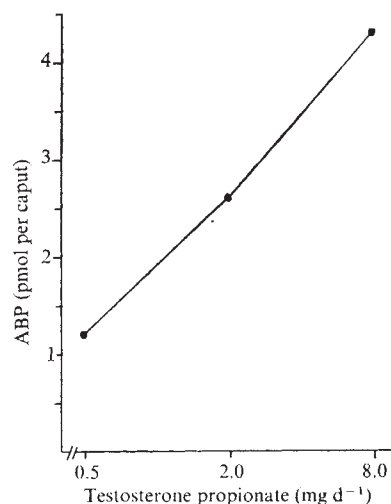


Fig. 1 Maintenance of ABP production by testosterone propionate (TP) in hypophysectomised rats. Male Sprague-Dawley rats in groups of four were hypophysectomised at 28 d of age and treated with various doses of testosterone propionate 0.5-8 mg d⁻¹ in sesame oil for 9 d, starting immediately after hypophysectomy. The animals were then killed on day 10, testis and capita epididymides weighed and homogenised in four volumes of buffer (50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA) and centrifuged at 105,000g for 1 h. The supernatants were diluted with equal volumes of the same buffer containing 20% glycerol and 60 nM ^3H -DHT. Concentrations of ABP was then examined by steady state polyacrylamide gel electrophoresis (SS-PAGE) as previously described¹².

tenance (treatment started immediately after hypophysectomy) and initiation (treatment started after regression of the germinal epithelium) of spermatogenesis, has been known for more than 30 yr (ref. 3), but no data have been provided which could explain this phenomenon.

We have shown that one action of FSH in the seminiferous tubules is to induce the production of a specific androgen binding protein (ABP) by the Sertoli cells^{4,5}. This protein is secreted through the rete testis fluid and concentrated in the caput epididymis⁶. We believe that the effect of FSH, mediated through ABP, is to amplify the testosterone stimulus to androgen-sensitive cells of the germinal epithelium. Since ABP is produced in the Sertoli cell⁷⁻⁹, we have suggested that this component can be used as a specific marker of Sertoli cell secretory function¹⁰. We report here that androgen alone is capable of maintaining Sertoli cell secretory function in immature hypophysectomised rats, but is not able to restore the secretory activity of the Sertoli cell after posthypophysectomy regression.

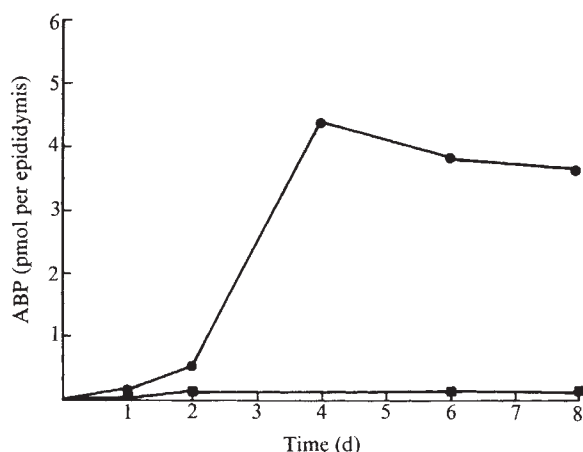


Fig. 2 Initiation of ABP production by FSH but not by testosterone propionate in hypophysectomised rats. Rats (groups of four) were hypophysectomised at 28 d of age, and their testes allowed to regress for 10 d before testosterone propionate treatment. Since 0.5 mg testosterone propionate daily was sufficient to maintain ABP in the capita epididymides for 10 d after hypophysectomy, animals were now treated with ten times this dose (■, 5 mg testosterone propionate daily) from 1–8 d starting at day 11 after hypophysectomy. ABP was measured in capita epididymides using SS-PAGE as above. Other groups of animals, hypophysectomised simultaneously, were injected with FSH alone (●, 250 μ g NIH-FSH-S₁₀ per day). FSH was dissolved in saline containing 0.1% bovine serum albumin and 0.25 ml was injected subcutaneously twice daily (125 μ g dose). TP was dissolved in sesame oil and 0.1 ml was injected intramuscularly once daily.

mised rats, but is not able to restore the secretory activity of the Sertoli cell after posthypophysectomy regression.

Figure 1 shows that androgen alone given to hypophysectomised animals is capable of maintaining Sertoli cell secretory function as measured by ABP levels in capita epididymides. Increasing doses of testosterone propionate gave a dose-dependent increase in the amount of ABP per caput (Fig. 1) as well as in testis weight (not shown). In the control animals (injected with oil only) no detectable ABP was present in the capita epididymides. Figure 2 shows that androgen alone is not capable of stimulating ABP production by the Sertoli cell within 8 d of treatment. Treatment with FSH, however, causes a rapid increase in ABP levels in the capita epididymides.

We conclude, therefore, that the Sertoli cell is responsive to androgens as well as to FSH (refs 4–6 and 12–14). Following regression of the cells for 10 d without hormone stimulation (hypophysectomy), however, FSH is required for full restoration of the secretory capacity. These studies also demonstrate the usefulness of ABP as a specific parameter of Sertoli cell secretory function.

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Occurrence of synapses in peripheral sensory nerves of arachnids

RECEPTOR cells of arthropods are presumed to send their axons directly into the central nervous system^{1,2}. Second order fibres supposedly do not occur in the periphery, and thus the first synaptic connections ought to lie within the ganglionic neuropiles. We report, however, that synapses do occur regularly in peripheral sensory nerves of at least two arachnid orders, whip spiders (Amblypygi) and harvestmen (Opiliones).

The first legs of whip spiders are modified appendages which are extremely long (20–25 cm) and slender (0.2 mm diameter). The tarsi are subdivided into about 100 segments and bear thousands of multi-innervated hair sensilla³. As there are no muscles within the tarsus, the two tarsal nerves consist of afferent fibres only (with the possible exception of some efferent fibres supplying epidermal glands).

Tarsi of the first legs of *Admetus pumilio* (Amblypygi) were prepared for electron microscopy and transverse sections at various segments (2, 4, 6, 8, 10, 13, 15, 17 and 60) were examined. Synapses were observed in most cross sections of the two tarsal nerves and occurred only on one or a few postsynaptic fibres (Fig. 1a), which are always located at the periphery of the nerve. Thus, the synapses are restricted to a very small area, while the surrounding thousands of axons comprising the leg nerve do not exhibit any junctional specialisation.

The postsynaptic fibre is relatively large. Short lateral extensions and the presence of ribosomes and many mitochondria suggest a branching dendrite. The presynaptic elements may form rather conventional synapses (Fig. 1a) or 'dyad' synapses⁴⁻⁹ (Fig. 1d). In the former case, there is

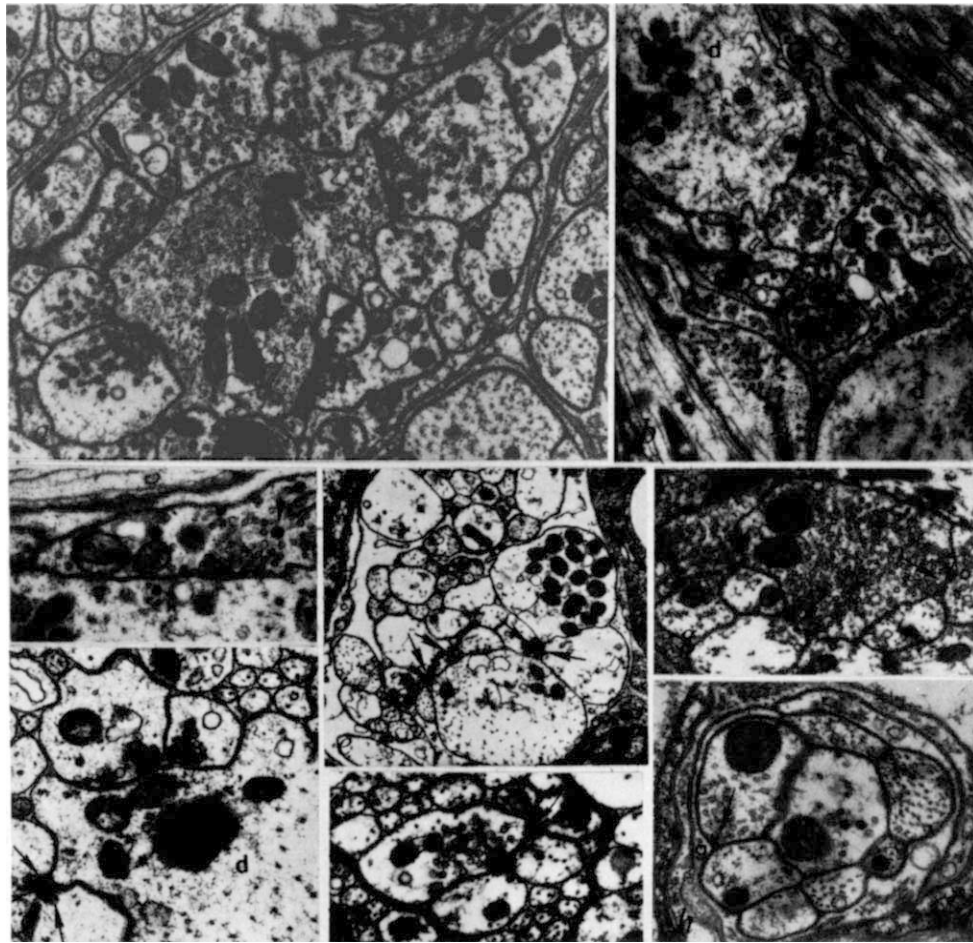


Fig. 1 *a-e*, *Admetus pumilio* (tarsus of leg 1). *a*, Part of a cross section from a tarsal nerve showing one relatively large dendrite (d), contacted by about ten presynaptic endings ($\times 15,400$). *b*, Para-sagittal section corresponding to Fig. 1*a*. The synapses are not evenly distributed along the long axis of the dendrite (d) but form clusters at regular intervals. Most synapses (arrows) contain round synaptic vesicles and presynaptic dense projections. Some terminals exhibit flattened synaptic vesicles (asterisk) ($\times 10,500$). *c*, Detail of a longitudinally sectioned terminal. The contact zone is delineated by a widened synaptic cleft. Several dense projections, a cluster of synaptic vesicles and few dense-core vesicles (arrow head) are typical for the presynaptic side. The postsynaptic side shows very little, if any, membrane thickening (compare with *a*) ($\times 18,350$). *d*, Part of a cross section of a tarsal nerve. Two presynaptic fibres form dyad synapses with a large dendrite (d). Another dyad synapse is indicated by arrows ($\times 22,800$). *e*, A bundle of intra-epidermal nerve fibres in cross section. Short extensions of two large fibres receive dyad synapses (arrows) ($\times 9,800$). *f-g*, *Phalangium opilio* (tarsus of leg 2). *f*, Cross section of a tarsal nerve showing typical dyad synapses. The small fibre on the right (long arrow) forms perhaps a reciprocal synapse with the large fibre (short arrow) ($\times 28,000$). *g*, Intra-epidermal nerve fibre, densely filled with synaptic vesicles, makes dyad synapses (arrows) with several adjacent fibres ($\times 15,700$). *h*, *Araneus diadematus* (tarsus of leg 1). Eight intra-epidermal nerve fibres in cross section. One fibre (arrow) exhibits synaptic vesicles and a dense plaque opposite the angle between the adjacent nerve fibre and a glial cell (g) ($\times 17,500$).

a high concentration of synaptic vesicles and a distinct synaptic cleft, yet little membrane thickening. The pre-synaptic side often has dense projections (Fig. 1*c*) but the postsynaptic side is only slightly thickened, if at all. The dyad synapse always involves three nerve fibres, usually two presynaptic axons and one postsynaptic dendrite. Both axon terminals have an electron-dense plaque on the cytoplasmic side of the membrane surrounded by a small cluster of synaptic vesicles. The typical arrangement of dyad synapses is shown in Fig. 1*d*.

Longitudinal sections show that the synapses do not cover the entire dendritic surface, but form clusters (Fig. 1*b*) at intervals of 6–8 μm . *En passant* synapses are commonly seen, which means that one presynaptic fibre often makes several synaptic contacts. Additional small synapses may be found in close proximity to the relatively large dendrites. From serial sections it seems that these contact dendritic extensions of the large fibre.

Synapses were also observed outside the tarsal nerves, within intra-epidermal fibre bundles (Fig. 1*e*). These also form either conventional or dyad synapses. In the latter case, several presynaptic fibres converge on a larger postsynaptic fibre.

The legs of harvest-men are especially long, and an investigation of the tarsal leg nerves in *Phalangium opilio* also revealed synapses, mostly of the dyad type (Fig. 1*f*) and seemingly not restricted to large postsynaptic fibres. One presynaptic axon often forms a dyad with two small postsynaptic fibres. This then represents a divergent synapse rather than the convergent type found in the whip spider. Definite synaptic contacts were also seen in intra-epidermal nerve fibre bundles (Fig. 1*g*).

A brief survey of the tarsus in a web spider (*Araneus diadematus*, Araneae) revealed synapses in the leg nerves as well as within intra-epidermal fibre bundles (Fig. 1*h*). These are, however, much less conspicuous and seemingly rarer than in the whip spider or harvest-man. Contacts similar to those shown in Fig. 1*h* have been reported in insect gustatory organs¹⁰, and have been interpreted as efferent and assigned a 'neurosecretomotor' function. This is also a possible explanation for the intra-epidermal synapses found in arachnids, but it could hardly be applied to the synapses within the leg nerves, because there are no effector organs except for some gland cells.

How can the presence of numerous, often considerably localised, synapses in sensory nerves of arachnid legs be

interpreted? At present, the origins of pre- and postsynaptic elements cannot be clearly determined and no definite answer can be provided. It seems likely, however, that the large dendrites belong to interneurons which receive the input of many primary receptors. At least in the case of the whip spider, the system could be comparable with the giant fibre system found in many invertebrates¹¹⁻¹³—except, of course, that the giant fibres lie within the central nervous system. In the whip spider the large fibres which receive synapses attain a diameter of 10 μm (proximal tarsal level), whereas the majority of sensory axons measure 0.2 μm or less. The large fibre diameter could provide a greater conduction velocity for nerve impulses; this may be an important feature, if one considers that most receptors are more than 20 cm away from the central nervous system. An additional advantage would be a reduction in the high number (10^4 – 10^5) of primary receptor axons.

From the large number of synapses occurring in peripheral sensory nerves, it must be concluded that some integration is taking place at the periphery of the arachnid nervous system. This may set the arachnids aside from insects, in which the first site of sensory information processing is believed to lie within the central nervous system².

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Neurological changes in fruit bats deficient in vitamin B₁₂

HAEMATOLOGICAL and neurological sequelae are the two major effects of vitamin B₁₂ deficiency in man. In the haemopoietic system, megaloblastic change leading to anaemia seems to be caused by deranged DNA synthesis¹. The cause of central nervous system demyelination, however, is unknown and progress in this area has been hampered by the lack of a suitable experimental animal model. We report that Egyptian fruit bats (*Rousettus aegyptiacus*), made vitamin B₁₂ deficient in captivity, develop neurological changes similar to those observed in human subacute combined degeneration of the spinal cord.

Previous attempts to find a suitable animal model for vitamin B₁₂ deficiency have proved difficult for several reasons. Herbivores obtain vitamin B₁₂ either by coprophagy² or, in the case of ruminants, from resident microorganisms in their upper gastrointestinal tract³. Consequently, it has been necessary to impose a variety of artificial procedures, such as the use of synthetic diets⁴, the prevention of coprophagy⁵ and antibiotics to eliminate gastrointestinal flora⁶, to produce vitamin B₁₂ deficiency in animals. Furthermore, animals made vitamin B₁₂ deficient by these procedures do not develop consistent haematological or neurological changes. The most convincing evidence of neurological change in vitamin B₁₂ deficient animals was reported in primates which developed

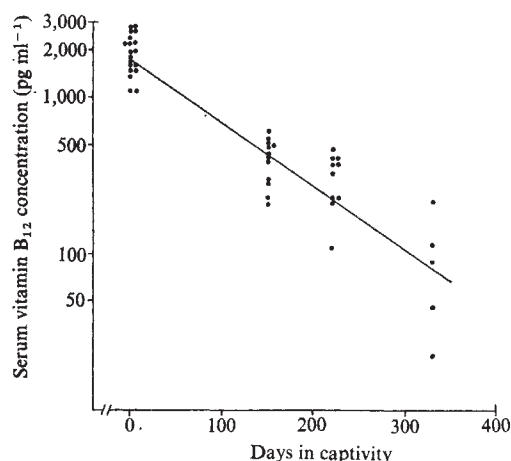


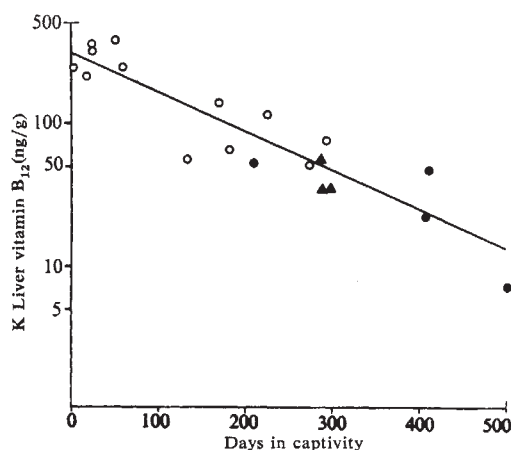
Fig. 1 Serum vitamin B₁₂ concentrations (log scale) at various times in 19 bats maintained on an all-fruit diet. The fall was exponential and highly significant after 330 d ($t_{22} = 14.8$, $P < 0.001$). The formula for the regression line was: $\log y = -0.0041x + 3.245$ and the calculated turnover rate [$(\ln 2/t_{1/2}) \times 100$] was 0.93% d⁻¹.

'cage paralysis' on a vegetarian diet⁷, but two recent studies^{8,9} failed to confirm these findings.

R. aegyptiacus (suborder Megachiroptera) is widely distributed in tropical and semitropical parts of the Old World. This species has been successfully kept in European zoos since the last century¹⁰, and its natural lifespan has been estimated to be 15–20 yr¹¹. We selected this species because its natural diet consists entirely of fruit and flowers¹⁰. Also, because of their habit of foraging on fruit trees remote from the caves in which they roost, faecal contamination of the diet is unlikely, and furthermore, coprophagy has not been observed. Although it is not known how these bats normally obtain their dietary supply of vitamin B₁₂, it is presumed that they may do so either by inadvertent ingestion of fruit pests, or by drinking stagnant water which may contain up to 1.0 ng vitamin B₁₂ ml⁻¹ (ref. 12). We excluded these potential sources of the vitamin in captivity without otherwise altering the bat's natural diet.

Adult male bats captured in the North-eastern Transvaal were maintained on a pest-free all-fruit diet consisting of washed and peeled bananas, papayas, pears and oranges fed *ad libitum*. Bats were supplied with clean tap water daily and their cage floor was kept clean. Blood samples were

Fig. 2 Liver vitamin B₁₂ concentration (log scale) in 19 bats which were killed at various times while on an all-fruit diet. The fall was exponential and highly significant ($t_{17} = 24.6$, $P < 0.001$). The formula for the regression line was: $\log y = -0.0029x + 3.061$ and the calculated turnover rate was 0.63% d⁻¹. Presence or absence of neurological changes shown by: ●, functional and structural abnormalities; ▲, functional, but no structural abnormality; ○, normal.



obtained by direct cardiac puncture for blood counts and measurement of the serum vitamin B₁₂ concentration using a radioisotope dilution method¹³. Liver vitamin B₁₂ concentration was measured by a modification of the serum method. Stained slides were prepared from rib bone marrow. Spinal cords were removed from freshly killed animals, sectioned and stained with haematoxylin and eosin, phosphotungstic acid haematoxylin, the Kluver-Barrera stain for myelin, Bielschowsky stain for axons and oil red O for lipids.

The serum vitamin B₁₂ concentration (mean $\pm$ s.e.) in 19 bats fed this diet fell significantly from 1964.0 ± 121.01 pg ml⁻¹ at the time of capture, to 98.6 ± 34.88 pg ml⁻¹ in five surviving bats after 330 d (Fig. 1). The liver vitamin B₁₂ concentration measured in bats killed at various times also fell significantly (Fig. 2). Over this period, there was no fall in the haemoglobin level, haematocrit or red cell count, nor any alteration in the calculated red cell indices. But the leukocyte count (mean $\pm$ s.e.) in 19 bats fell significantly from 19.5 ± 1.11 to 4.9 ± 0.38 thousand μ l⁻¹ in 12 surviving bats after 130 d ($t_{29} = 12.5$, $P < 0.001$). This fall reflected a decrease in both granulocytes and non-granulocytes, but no further significant change was observed beyond 130 d. Injection of 200 ng vitamin B₁₂ per week in two bats resulted in a return of the leukocyte count to initial values. There was no increase in polymorphonuclear leukocyte lobe count, nor was there bone marrow evidence of megaloblastic change in either myeloid or erythroid precursors.

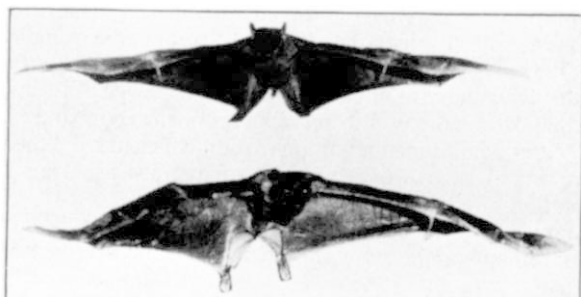


Fig. 3 Single representative frames of (a) normal and (b) deficient bats, taken during middle of downstroke. Three major functional alterations occur in the flight pattern: Gross irregularities of the surface of the dactylopatagium result in massive turbulence and partial stalling; the irregular surface of the tip negates the propulsive force normally arising from the downward twist of this region; slackening of the trailing edge (normally held taut between foot and fifth digit) increases drag.

After 200 d, as bats became progressively more vitamin B₁₂ deficient (Fig. 2) seven of the ten surviving animals developed ataxia, manifested by difficulty in climbing. This seemed to result from a loss of reflex proprioceptive sensation in the lower extremities, as bats were unable to disengage their claws from the wire mesh during climbing.

Changes in the normal flying cycle were also noted, and were analysed by high speed cine and still photography. The most obvious abnormalities occurred during the downstroke, when the dactylopatagia were crumpled and irregular compared with the normally smooth, fully extended wing surface (Fig. 3). Full separation of the phalanges was lost, resulting in laxity of the individual membranes. At the beginning of the downstroke, when usually the phalanges are fully extended and radially deviated, those of the deficient bats trailed in the slipstream. The relatively coarser control of the shoulder, elbow and thumb was little affected but the weakened fifth digit led to slackness of the trailing edge of the plagiopatagium. The upstroke, being partly a passive phenomenon and requiring little precise aerodynamic control of the distal wing, was minimally affected.

Histological sections of the spinal cords of bats killed after 200 d and stained for myelin, showed patchy spongiose change

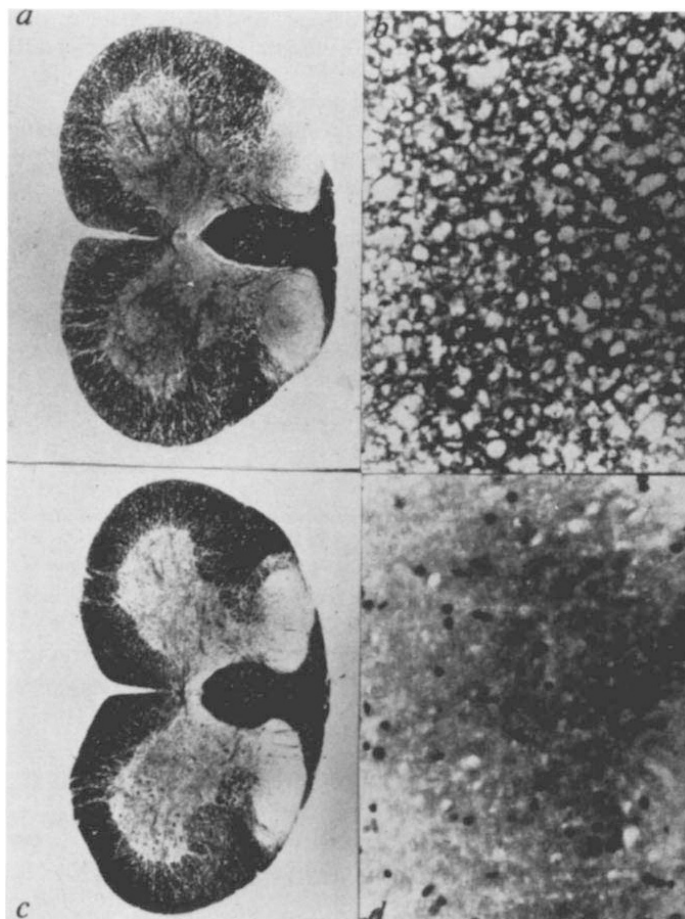


Fig. 4 Histological sections of the spinal cord taken from the upper thoracic region of fruit bats. Sections stained with Kluver-Barrera stain for myelin. a, Low power view ($\times 14$) of section from a vitamin B₁₂ deficient bat (serum vitamin B₁₂ concentration 108 pg ml⁻¹; liver vitamin B₁₂ concentration 54 ng g⁻¹) obtained 210 d after receiving an all-fruit vitamin B₁₂ deficient diet. The white matter shows patchy spongiose change affecting mainly the lateral and ventrolateral columns. b, High power view ($\times 259$) of the white matter from the same section (deficient bat). c, Low power view ($\times 14$) of section from a normal bat (serum vitamin B₁₂ concentration 2,340 pg ml⁻¹; liver vitamin B₁₂ concentration 320 ng g⁻¹). d, High power view ($\times 259$) of the white matter from the same section (normal bat). The section from the normal bat does not show the patchy spongiose change seen in the spinal cord of the vitamin B₁₂ depleted animal.

in the white matter of the lower cervical and upper thoracic region, mainly affecting the lateral and ventrolateral columns and suggestive of early demyelination (Fig. 4). The changes were observed in four out of five bats which had developed evidence of functional neurological impairment; their liver vitamin B₁₂ concentrations are shown in Fig. 2. The lesions are similar to those lesions found in vitamin B₁₂ deficient humans except that histological changes in man affect mainly the dorsal and lateral columns¹⁴. There was no evidence of reactive astrocytosis or gliosis, and the stain for axons showed no differences compared with normal cords. No lipid-containing phagocytes were observed on an oil red O stain. Bats given 200 ng cyanocobalamin per week by intramuscular injection did not develop any neurological changes, but cyanocobalamin supplements failed to reverse functional changes once they had appeared.

In summary, we have shown that the fruit bat can be rapidly depleted of vitamin B₁₂ when kept on a diet similar to its natural one and that fruit bats may be used as an experimental model to study the neurological changes produced by vitamin B₁₂ deficiency in the absence of anaemia. Further studies are in progress on the nature of these neurological changes.

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Evidence for the B₁₂-dependent enzyme ethanolamine deaminase in *Salmonella*

THE enteric bacteria *Escherichia coli* and *Salmonella typhimurium*, like man, do not synthesise significant amounts of cobalamin (B₁₂) compounds and thus depend on exogenous vitamin B₁₂ for their B₁₂-dependent enzymes¹. In *E. coli* and *S. typhimurium* the only reported B₁₂-dependent enzyme catalyses the final step in methionine biosynthesis—the methylation of homocysteine to give methionine. These bacteria do not depend on exogenous B₁₂ for growth, however, for they have an alternative B₁₂-independent homocysteine transmethylase (non-B₁₂ enzyme) which can catalyse the same reaction. The non-B₁₂ transmethylase is much less efficient than the corresponding B₁₂-dependent enzyme: in bacteria grown in the absence of vitamin B₁₂, the non-B₁₂-dependent enzyme comprises 3-5% of the total soluble protein². If this enzyme is blocked by mutation, the mutant bacteria require either methionine itself or exogenous B₁₂, which allows them to utilise the B₁₂-dependent enzyme for methionine biosynthesis³. We have sought other B₁₂-dependent enzymes in *S. typhimurium* and found evidence for an ethanolamine deaminase. We did not find other examples of parallel non-B₁₂-dependent and B₁₂-dependent enzymes in *S. typhimurium*. *E. coli* also seems to have an ethanolamine deaminase.

We first searched for B₁₂-dependent catabolic enzymes, since other bacteria use such enzymes in major catabolic pathways for arginine, lysine, ornithine, propanediol, glycerol, glutamate and nicotinate⁴. If *S. typhimurium* or *E. coli* have similar B₁₂-dependent catabolic pathways, they would have gone unnoticed in surveys of utilisable carbon and nitrogen sources performed in the absence of exogenous B₁₂. Petri dishes of carbon-deficient or nitrogen-deficient medium were seeded with about 2 × 10⁸ cells of wild type *S. typhimurium* LT 2 and a few µg of each test compound was added to the surface of the plate and incubated at 37 °C (ref. 5). Each substance was tested in the presence or absence of vitamin B₁₂ (cyanocobalamin, 0.4 µg ml⁻¹). Growth was observed daily for a week. Ethanolamine was found to serve as a source of nitrogen in the presence of vitamin B₁₂.

With B₁₂, ethanolamine supported as much growth as did ammonium. The final growth yields were the same, regardless of whether ethanolamine or NH₄Cl was used as the nitrogen source in a minimal medium containing vitamin B₁₂, glucose and

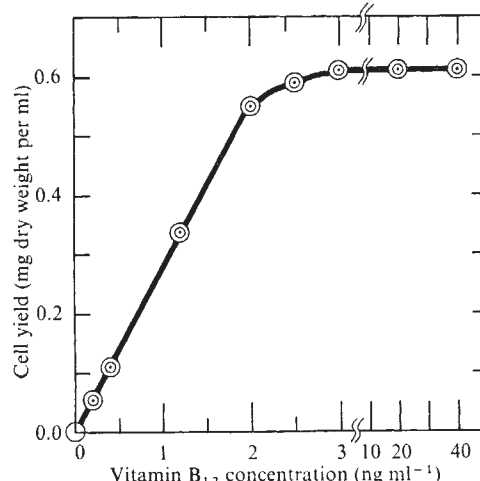


Fig. 1 Growth of *Salmonella* on ethanolamine and vitamin B₁₂. The growth medium was T2 salts mixture⁹ supplemented with 0.5% glycerol, 7.4 mM ethanolamine hydrochloride, and the indicated quantities of vitamin B₁₂. Cultures were shaken for at 37 °C. After 34 h of incubation, cell yields were determined by measuring the 650 nm absorbance of cell suspensions.

salts. With ethanolamine, the doubling time was about 100 min, with NH₄Cl, about 50 min.

Vitamin B₁₂ was essential for the utilisation of ethanolamine. At low B₁₂ concentrations, the growth of *S. typhimurium* was proportional to the concentration of vitamin (Fig. 1). Cobaltous chloride did not satisfy the vitamin B₁₂ requirement, indicating that the synthesis of B₁₂ compounds in *Salmonella* is not limited by the availability of cobalt.

Ethanolamine may also serve as a poor carbon source with B₁₂. After 36 h of incubation on carbon-deficient solid medium, there was slight growth around filter paper disks saturated with a solution of ethanolamine.

Many members of the Enterobacteriaceae may be able to utilise ethanolamine as a nitrogen source in the presence of exogenous vitamin B₁₂. *E. coli* (K12) and *Enterobacter aerogenes* (ATCC 1033) showed the same response as *S. typhimurium* to vitamin B₁₂ and ethanolamine. Turner *et al.* have reported⁶ that *Erwinia* spp. utilise ethanolamine as a nitrogen source, but there is no specific evidence for a B₁₂-dependent pathway.

We next used penicillin selection procedures to determine whether homocysteine transmethylase is the only system in which *S. typhimurium* has alternative B₁₂-dependent and non-B₁₂-dependent enzymes. If other systems are catalysed by alternative B₁₂-dependent and non-B₁₂-dependent enzymes, it should be possible to inactivate the latter enzymes by mutation; mutants lacking the non-B₁₂-dependent enzyme should require exogenous B₁₂ for the corresponding B₁₂-dependent enzyme. Mutants were induced by saturating a fully grown culture of *S. typhimurium* LT 2 with diethylsulphate and incubating it for 10 min. A sample (0.1 ml; 2 × 10⁸ cells) of this culture was then transferred to 5 ml of nutrient broth and grown overnight. Then 0.1-ml portions of the culture were collected on Millipore filters and subjected to selection⁷. After treatment with penicillin, the cells were filtered, washed and grown in a minimal glucose medium supplemented with vitamin B₁₂. Clones derived from these cultures were tested for their ability to grow on minimal glucose medium with and without methionine or vitamin B₁₂ supplements. All incubations were at 37 °C. From 217 survivors of the selection procedure, we obtained 89 B₁₂-requiring mutants, all of which grew in the presence of either vitamin B₁₂ or methionine. They were presumably all *metE* mutants which lack the non-B₁₂ enzyme in the methionine pathway.

We then modified the penicillin selection procedure to exclude methionine mutants. Methionine (0.2 mM) was added to the penicillin solution. Any methionine auxotrophs would then be killed by the penicillin. Any B₁₂-requiring mutants which do not respond to methionine, would presumably have lost the non-B₁₂

part of some metabolic system which normally has alternative B_{12} -dependent and non- B_{12} -dependent enzymes. We obtained no mutants of this type, even after two sequential selection operations on four mutagen-treated cultures. This technique should have yielded mutants occurring at frequencies as low as 10^{-8} per mutated cell.

The failure to obtain mutants in the second series of penicillin selection experiments suggests that only the methionine biosynthetic system has alternative B_{12} -dependent and non- B_{12} -dependent enzymes. Other explanations for our failure to isolate mutants could be: (1) the penicillin selection procedure was not as efficient as expected; (2) the traces of B_{12} found in *S. typhimurium* were sufficient for growth, thus preventing the isolation of B_{12} auxotrophs; or (3) the systems using alternative B_{12} -dependent and non- B_{12} -dependent enzymes are not needed for growth under laboratory conditions. The first explanation is unlikely, however, since so many B_{12} -requiring methionine mutants were isolated in the first selection experiments. The second explanation is unlikely unless the hypothetical B_{12} enzyme has a much higher affinity for B_{12} and is required in much smaller amounts than the methionine biosynthetic enzyme.

Our nutrition experiments suggest that *S. typhimurium* and *E. coli* have a B_{12} -dependent ethanolamine deaminase (ethanolamine-ammonia-lyase, EC 4.1.3), analogous to the enzyme in clostridia⁶. It seems pertinent that these organisms have two B_{12} -dependent metabolic pathways and yet they cannot synthesise significant amounts of the B_{12} -compounds. Under the usual laboratory conditions, neither pathway is essential for growth: methionine can be made by the B_{12} -independent pathway and ethanolamine is not usually needed as a nitrogen source. Nevertheless, the fact that the bacteria have both pathways suggests that they are at times advantageous and that the bacteria often have exogenous supplies of B_{12} compounds. This is not surprising, since faeces and sewage are rich sources of vitamin B_{12} and related compounds.

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zine (CP), dissolved in normal saline, were injected intraperitoneally into 793 male Osborne-Mendel rats (150-175 g) which had been double-ligated below the renal arteries^{3,4}. Beginning on the fifth day after ligation, the animals were pretreated with daily injections of one drug for 3 d. On the eighth day after ligation, 8 h after the last injection, half the animals were given a dose of 5-HT (10 mg kg⁻¹) and half were given noradrenaline (3.25 mg kg⁻¹). Twelve hours after the 5-HT or 5 h after the noradrenaline they were bled terminally into preheparinised syringes by cardiac puncture under light pentobarbital anaesthesia. The blood was cooled and cold-centrifuged at 17,750g for 2 min, and the plasma was pipetted off for enzyme analysis.

For glutamic-oxaloacetic transaminase (GOT) we used the Reitman-Frankel method (U ml⁻¹); for lactic dehydrogenase (LDH), the Babson-Phillips method (IU); for creatine phosphokinase (CPK), the Rosalki method (μU ml⁻¹), and for alkaline phosphatase, the Babson-Phillips method (U ml⁻¹)⁴. The drugs were given to groups of animals in scaled doses: IP (297 animals) 1.25, 2.5, 5, 10, 20 mg kg⁻¹; CP (301 animals) 2.5, 5, 10 mg kg⁻¹; PBZ (195 animals) 2.5, 5, 10, 20 mg kg⁻¹.

Immediately after ligation the first four plasma enzymes increased prominently but all returned to normal by the fifth day⁴ (40 animals). 5-HT or noradrenaline in the doses used, when not preceded by ligation of the aorta, caused no significant increase in enzyme activity⁴. Peak increases occurred in ligatured animals 12 h after 5-HT (ref. 4) and 5 h after noradrenaline (determined in this study using 52 animals analysed 1, 2, 3, 4, 5, 8, 12 and 16 h after injection). In neither the test-control group nor any of the ligatured animals given IP, PBZ or CP before 5-HT or noradrenaline was there a significant increase in alkaline phosphatase, suggesting that liver damage was not the cause of plasma GOT, GPT, CPK and LDH increases. For the purpose of this study we therefore term them muscle enzymes. Injections of ligatured animals for 3 d with the largest doses of IP, PBZ or CP alone did not alter plasma enzyme values. Animals receiving IP and PBZ (housed 3-5 per 27 × 44 cm plastic box) were not noticeably more active or less active than controls, but those given CP were slightly sluggish.

The increase in all four plasma muscle enzymes provoked by a single dose of 5-HT was prevented, in a consistent relation to dose, by prior IP, PBZ or CP (Figs 1-3); the most complete effect was with IP (10 and 20 mg kg⁻¹), subsequent doses of 5-HT having almost no effect on enzyme activity (Fig. 1). The increase in muscle enzymes was prevented in a similar manner when PBZ and CP were given before noradrenaline (Figs 2 and 3). IP before noradrenaline, however, had two opposite effects, depending on the dose: small doses (1.25 and 2.5 mg kg⁻¹) significantly prevented the induced increase of all the enzymes, while the largest dose (20 mg kg⁻¹) markedly enhanced the increase of CPK and LDH (and had no net effect on GOT and GPT). Thus, the largest dose of IP had opposite effects on the presumably indirect muscle-damaging actions of 5-HT and noradrenaline (Fig. 1).

The muscle damage caused by low doses of 5-HT or noradrenaline given to aorta-ligated rats is probably the result of ischaemia^{1,3}. The protective effect of pretreatment observed with PZB or CP before noradrenaline or 5-HT may be related to the known ability of both to block the action of α-adrenergic agents as well as responses to 5-HT⁶⁻⁷. The enhancing effect of high doses of IP on muscle damage induced by noradrenaline may be related to the known ability of IP to reduce uptake of noradrenaline into nerve terminals^{5,8}. Since IP also reduces such uptake of 5-HT^{9,10}, the reason for its action in blocking the 5-HT effect in our animals at all doses and the noradrenaline effect in low doses must be explained otherwise, perhaps related to the following: IP often causes orthostatic hypotension and it can antagonise or reverse the local vasoconstrictor action of noradrenaline⁶, presumably by α blocking (although desmethyl-IP can block release of noradrenaline from nerve endings⁶); and it can block the spasmogenic effects of 5-HT on the smooth muscle of isolated guinea pig ileum⁶. It is possible that, at certain IP doses, analogous mechanisms predominate in the

Drugs blocking the muscle-damaging effects of 5-HT and noradrenaline in aorta-ligated rats

USING an animal system proposed as a model for the hypothesis that Duchenne muscular dystrophy might be caused by an impaired blood supply within muscle¹⁻³, we have found that certain drugs prevent the muscle damage typical of this disease. The model involves combining ligation of the aorta with a minimal dose of serotonin (5-hydroxytryptamine, 5-HT) or noradrenaline to produce myopathy of the lower extremities, which is evident in histochemical changes³ and increased amounts of some muscle enzymes in the plasma⁴.

Imipramine (IP), phenoxybenzamine (PBZ) or chlorproma-

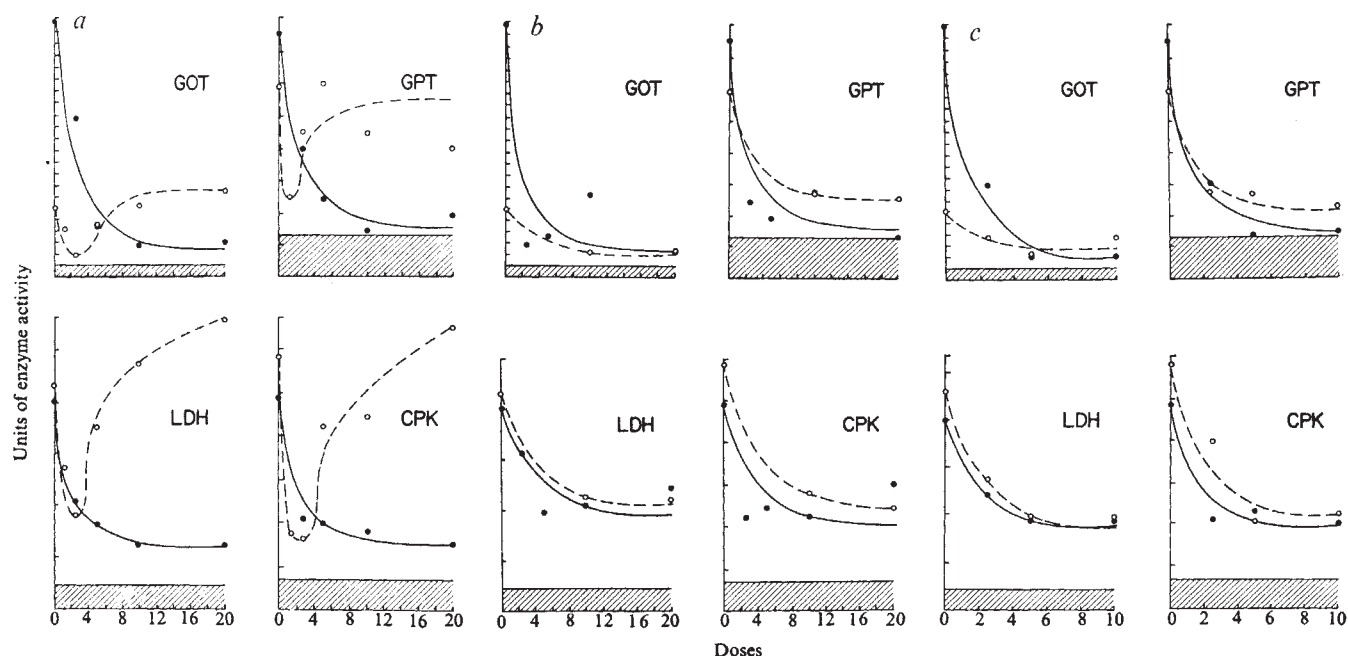


Fig. 1 Mean values (40–75 animals for each point) of plasma enzyme levels of GOT, GPT, LDH and CPK in aorta-ligated rats given a single dose of 5-HT (●) or noradrenaline (○), pretreated by: *a*, imipramine; *b*, phenoxybenzamine; or *c*, chlorpromazine. Doses of pretreatment drugs on abscissa are given in mg kg^{-1} . Divisions on the ordinate are GOT 100, GPT 20, LDH 100 and CPK 100 U of enzyme. Zero point on the abscissa indicates enzyme level in control animals ligatured and given 5-HT or noradrenaline but no pretreatment drug; cross-hatched region indicates maximal upper range (on ordinate) of enzyme level in normal ligature-only rats, which is the same as in normal rats.

smooth muscle of the abnormal (underperfused) intramuscular arterial vessels of our model.

We conclude that this is a useful animal test system for evaluating interaction of drugs which affect muscle, presumably by acting on the intramuscular arterial system. If the muscle-damaging mechanism in our model is, in principle, analogous to that in Duchenne muscular dystrophy, these experiments provide screening data of possible clinical relevance. Because local metabolic factors are thought to be producing maximal vascular deconstriction in ischaemic regions of normal muscle⁷, blockers of amine-induced vasoconstriction in normal persons may only be able to open collateral feeding vessels. In Duchenne dystrophy muscle, however, we propose that there could be an inherited defect making small arterial vessels hyposensitive to deconstrictive local metabolic factors and/or overly sensitive to other constrictive ones such as vasoactive amines, defects for which amine-blockers might be at least partially corrective.

The pertinence of our model, involving aorta ligation and vasoactive amine in low dosage, to Duchenne dystrophy has recently been questioned and an alternative one proposed—IP plus 5-HT at very high doses (10 times that used in our experiments) in non-ligated rats¹¹. Pretreatment with IP had only a worsening effect (histologically) on muscle damage produced by the very high doses of 5-HT¹¹. Nevertheless, in Duchenne dystrophy patients we favour the mechanism/model of a small amount of vasoactive substance interacting with an underlying arterial vessel abnormality because they have no systemic evidence of large circulating amounts of vasoactive amine.

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Fluorescence techniques for following interactions of microtubule subunits and membranes

THERE is increasing evidence that several cell functions are controlled by the state of polymerisation of microtubules (MTs) and by the interaction of MTs with membranes. For example, previous studies in our laboratory have shown that colchicine-sensitive structures, presumably MTs, determine the topographical organisation of cell membrane components^{1–3}. Conditions for the polymerisation of MTs *in vitro* have been described⁴. The MT subunit has been identified by centrifugation studies as a 6S dimer of approximately 110,000 daltons⁵. An additional 36S component is required for MT assembly⁶. The established procedures used, however, to record MT polymerisation (light scattering and viscosity measurements supplemented by negative staining and electron microscopy) do not reveal details of the interactions between subunits undergoing polymerisation and cannot be applied in the presence of elements such as membranes that contribute separately to light scatter and viscosity.

We report here a new method to monitor MT polymerisation and to investigate interactions between MTs and membranes. Our approach involves long range resonance energy transfer between different fluorescent moieties separately conjugated to different MT subunits or to membranes. The method permits study of weak or readily dissociable interactions between membranes and MTs or between subunits, which may not

survive techniques that physically separate or dilute the complexes.

Resonance energy transfer occurs when a chromophore which is excited by the absorption of energy transmits the energy to another (acceptor) chromophore some distance away. This transmission requires the overlap of the emission spectrum of the first (donor) chromophore with the absorption spectrum of the acceptor, but it does not involve the actual reabsorption of light by the acceptor. It is therefore referred to as non-radiative or resonance energy transfer. Resonance transfer requires that the distance between the chromophores be relatively close (not exceeding 100 Å) and in fact quantitative theory allows one to estimate the distance between chromophores from the extent of energy transfer⁷⁻¹⁰.

In our experiments fluorescein isothiocyanate (FITC) was used to label one population of MT subunits (protein dimers called tubulin and designated T) or membranes, and rhodamine isothiocyanate (RITC) was used to label a second population of tubulin. These chromophores were chosen because they bind covalently and because the emission spectrum of fluorescein (donor) extensively overlaps the absorption spectrum of rhodamine (acceptor). In the non-polymerised state a mixture of fluorescein-labelled (F-T) and rhodamine-labelled (R-T) tubulins does not show resonance transfer. With polymerisation (or aggregation) of MT-subunits, however, the chromophores are brought sufficiently close together so that energy transfer occurs. The degree of transfer is thus dependent on the number of units polymerised. Figure 1 shows a time course of the polymerisation of MTs followed by light scattering and resonance energy transfer. The progressive increase in scatter was the same whether or not dye was conjugated with the tubulin, indicating that these low concentrations of bound chromophores do not interfere with MT assembly. At the same time resonance transfer of energy from fluorescein to rhodamine was measured by exciting the sample at 480 nm, and recording the emission spectrum between 500 and 620 nm (to include

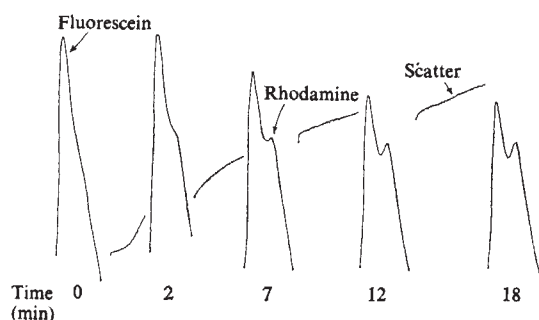


Fig. 1 Time course of light scattering and fluorescence emission spectra during MT polymerisation. Tubulin (T) was purified from rabbit brain by successive cycles of polymerisation and depolymerisation as described by Shalanski¹¹. Protein-dye conjugates were formed by incubation of purified protein (4 mg ml⁻¹) with fluorescein isothiocyanate (FITC) or rhodamine isothiocyanate (RITC) for 30 min at 4 °C. Incubation was stopped by separating the conjugates from the free dye by column chromatography (Sephadex G-25). Dye to protein molar ratios (D/P) were determined by absorbance measurements using extinction coefficients of the bound dyes¹² and protein measurements by the method of Lowry¹³. The absorbances of the mixtures followed spectroscopically did not exceed 0.20 absorbance units. In this experiment incubations of 10 µg dye per mg protein and 5 µg mg⁻¹ protein for RITC and FITC respectively gave approximate D/P values of 0.35 for R-T and 0.08 for F-T. Tyndall scatter was measured at 650 nm using parallel polarised light, monitored at 90° to the incident beam. (This assures a high signal to noise ratio.) Viscosity changes (not shown) agreed closely with the time course of scatter measurements. At intervals the latter were interrupted and emission spectra obtained by exciting the sample at 480 nm and scanning from 500 to 650 nm. Corrected spectra were obtained using a Perkin-Elmer MPF4 fluorometer. At zero time a single peak representing only fluorescein emission was obtained. A rhodamine emission peak developed progressively as resonance energy transfer occurred between the polymerising fluorescein and rhodamine-labelled subunits. See text.

both fluorescein and rhodamine emissions). The sample was excited at 480 nm instead of at the excitation maximum of 496 nm to minimise the direct excitation of rhodamine. Before polymerisation (Fig. 1, zero time) a simple fluorescein emission spectrum was observed. There was no energy transfer and thus no rhodamine emission. Later (Fig. 1, 2 min and later) as subunits separately labelled with F and R were brought together, resonance transfer occurred and the characteristic emission peak of rhodamine progressively increased. Simultaneously, since resonance transfer and emission compete for the excited state, a decline in fluorescein emission was observed. When F-T alone (no R-T present) were polymerised, no decrease in fluorescein emission occurred. The depopulation of the fluorescein excited state by energy transfer to rhodamine is also reflected in a decrease in the excited state lifetime. Fluorescent lifetime measurements¹⁴ showed a decrease from 5.15 ns for MT assembled from F-T dimers alone to 3.64 ns for maximally (> 70%) polymerised MTs assembled from the F-T plus R-T mixture. From these data a mean distance between chromophores of 44 Å was calculated⁷. The same distance was obtained by calculation from the energy transfer spectrum of maximally polymerised MT obtained in Fig. 1, and is consistent with the known geometry of MTs (Fig. 2).

Energy transfer did not occur when depolymerised F- and R-labelled subunits were incubated under conditions in which polymerisation did not occur (at 4 °C, or in the presence of 4 mM calcium chloride, or in the absence of GTP). But we observed interactions between subunits in the presence of colchicine, a plant alkaloid that binds with tubulin subunits and is thought to prevent their association into microtubules. A tenfold molar excess of colchicine slowed markedly the increase in the light scattering. None the less, in the presence of colchicine resonance transfer increased progressively with time and to nearly the same degree as in the absence of the alkaloid. This indicates that most of the MT subunits are probably associated into small aggregates. These aggregates seem to be readily dissociable: the addition of unlabelled tubulin to the colchicine mixture, or simple dilution, led to a rapid fall in energy transfer. In contrast, neither unlabelled tubulin nor dilution altered the energy transfer of polymerised MT. Thus, the effect of colchicine on the interaction of subunits was different from the effects of cold or calcium which prevent polymerisation and the development of energy transfer and light scattering.

Preliminary experiments have also demonstrated interactions of MT and/or tubulin with isolated cytoplasmic membranes. Cytoplasmic membranes were isolated from rabbit polymorphonuclear leukocytes as previously described⁹ and incubated with FITC at approximately 25 µg FITC per 100 µg membrane protein for 10 min at 4 °C. The labelled membranes were then washed free of unreacted FITC by recentrifugation through a discontinuous sucrose gradient. R-T was prepared as before and mixed with the labelled membrane. At 4 °C virtually no energy transfer took place between F-labelled membranes and R-labelled tubulin. At 37 °C polymerisation of R-T occurred, and resonance energy transfer between F-membranes and R-tubulin developed in parallel with the increase in light scatter. This transfer was not diminished by the addition of unlabelled tubulin subsequent to polymerisation. Similar experiments using rhodamine-labelled bovine serum albumin and fluorescein-labelled membranes did not reveal energy transfer at any temperature. These experiments suggest a specific association of polymerised MT with membrane. Energy transfer between R-T and F-membranes increased progressively with time in the presence of colchicine, just as it did between R-T and F-T with colchicine. Further, addition of unlabelled tubulin to the membrane-tubulin complex formed in the presence of colchicine (10⁻⁴ M) rapidly reduced the resonance transfer. Thus, in the presence of colchicine the association of tubulin with membranes, like the association of tubulin with tubulin seems readily reversible, and is distinct from the association formed in the absence of the alkaloid. The high

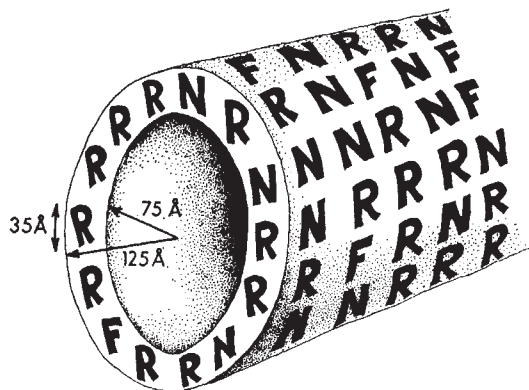


Fig. 2 Schematic diagram of microtubule and disposition of constituent fluorescent labelled subunits. The diagrammatic tubule was generated using the known geometry of MTs¹⁵ and distributing tubulin dimers labelled with fluorescein (F), rhodamine (R) and unlabelled dimers (N) in the tubule according to a list of random numbers. The proportions of F, R and N were calculated from the D/P ratios of F-T (0.15) and R-T (0.46) measured in a maximally (> 70%) polymerised suspension of MT, assuming that the association of dye molecules with tubulin follows a Poisson distribution¹⁶. In this mixture, the distance between F and R calculated from resonance transfer as described in the text is approximately 40 Å. This is consistent with the dimensions of the MT as shown, which is made up of protein dimers with a cross-sectional diameter of roughly 40 Å. This correspondence suggests that resonance transfer occurs only between immediately adjacent subunits.

efficiency of energy transfer observed (> 50%) indicates considerable interaction of the tubulin and membrane, and suggests that some labelled constituent of the membrane preparation could serve as a nucleation centre for MT polymerisation.

In summary the use of long range non-radiative energy transfer provides a new approach to the study of MY polymerisation. This method gives information on the geometry and interactions of subunits and of interactions between MTs and other cell constituents. It has been applied to demonstrate loosely associated tubulin aggregates formed in the presence of colchicine, and binding of microtubules to isolated membranes, neither of which has been observed with conventional techniques. These experiments are now being extended to explore the specificity and number of membrane binding sites for tubulin under different physiological conditions. The approach should prove useful in the study of other polymerisations (for example, microfilaments), particularly of their interactions in mixed systems containing membranes.

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Lateral compressibility and penetration into phospholipid monolayers and bilayer membranes

THE phospholipid bilayer¹ in certain biological membranes is maintained so that the range of temperature over which the gel-to-liquid crystal transition occurs includes the environmental temperature². As a result, clusters of phospholipid molecules in crystalline and liquid-crystalline states¹ coexist in the membrane and the isothermal lateral compressibility of the membrane lipids is enhanced³. Any increase in compressibility should facilitate insertion of foreign molecules into the bilayer thereby affecting transport across the membrane. Indeed, there is evidence that transport of ions^{4,5} and sugars³, and penetration of an enzyme⁶ is increased when the chain-melting transition of the lipids occurs. The lateral compressibility of phospholipid bilayers has not been measured, however, and there is no direct evidence for an increase in compressibility at the point where the gel-to-liquid crystal phase transition occurs. In order to measure lateral compressibility, compression solely in the plane of the bilayer is required. Such directed compression of bilayers will be difficult but it can be readily achieved with monolayers at the air-water interface. Since the molecular packing in such monolayers of phospholipids is equivalent to that in bilayers dispersed in excess water^{1,7}, this model system is particularly convenient for exploring the role of lateral compressibility. Here we show (1) how the compressibility of lecithin monolayers varies with packing density and changes at the chain-melting transition, and (2) how penetration of a hydrophobic protein is dependent on the compressibility.

The procedures used to obtain the surface pressure (π)-molecular area (A) isotherms for chromatographically pure 1,2-dipalmitoyl phosphatidylcholine (lecithin) have been described before^{7,8}. The preparation and adsorption characteristics of 1-¹⁴C-acetyl- β -casein A have also been published^{9,10}: this protein is very hydrophobic and its behaviour at the air-water interface is like that of proteins which are associated with lipids *in vivo* and quite different from that of highly water-soluble globular proteins. The penetration experiments were performed at constant surface area by maintaining the lecithin monolayer at an initial A with a barrier and injecting the protein into the substrate. The initial substrate concentration of protein was 1×10^{-4} g per 100 ml and the changes in π and surface concentration of protein (Γ) were monitored during adsorption with a Wilhelmy plate and gas-flow counter¹¹, respectively.

Figure 1a shows the π - A curves for dipalmitoyl lecithin spread at 20 °C and 30 °C on to a phosphate buffer ($I = 0.1$, pH 7); the isotherms are similar to those for a 0.1 M NaCl substrate⁷. The inflection at $\pi = 5.6$ mN m⁻¹ and $A = 77$ Å² per molecule (this point is difficult to reproduce and is sensitive to the rate of compression) is a result of the onset of two-dimensional crystallisation^{1,7} and over the region where the π - A curve is more-or-less horizontal the monolayer consists of a mixture of crystalline and liquid-crystalline domains. Because the transition is equivalent to that occurring in bilayers, the critical temperature^{1,7} for the monolayer transition is the same as the bilayer transition temperature (41 °C). The monolayer phase transition is probably best described as "diffuse" first order¹²⁻¹⁶ because the compressibility ($C = -1/A(\delta A/\delta \pi)_T$) plotted as a function of A (Fig. 1b) does not become infinite at the onset of condensation¹⁷. The form of the plot of C against A is the

same as that generally found for monolayers which undergo the phase transition¹⁷. The dependence of C on the phase of the dipalmitoyl lecithin monolayer is similar to that generally found¹⁶ for lipids: thus C increases by a factor of about 5–0.15 m mN⁻¹ on moving from the liquid-expanded (for a definition of terms see ref. 18) to the transition (intermediate¹⁸) region. In the crystalline (condensed) region $C \sim 0.004$ m mN⁻¹.

The magnitude of C is directly related to fluctuations in the total number of particles in the system¹⁹. The high compressibility at the phase transition results from large density fluctuations in this region. Because of such fluctuations in surface density π will also fluctuate and Blank²⁰ has shown that the instantaneous surface pressure is related to the mean value $\langle \pi \rangle$ by equation (1).

$$\pi \simeq \langle \pi \rangle [1 \pm (kTC/A)^{1/2}] \quad (1)$$

As a consequence of the enhanced lateral compressibility in the transition region, when $\langle \pi \rangle \sim 20$ mN m⁻¹ (which is characteristic of phospholipid bilayers¹) π can fluctuate locally between 3 and 37 mN m⁻¹. Fluctuations which give rise to a low π are equivalent to hole formation in the monolayer; such holes lead to enhanced permeation of small molecules^{20, 21}.

High values of C also enhance penetration of large molecules such as proteins which compress the lipid to clear a space (X) for themselves. In this case, penetration is opposed by an activation energy barrier $E = \langle \pi \rangle X$. Penetration continues with increasing $\langle \pi \rangle$ (Fig. 1c) until E becomes too large; generally this point is reached and penetration ceases when $\langle \pi \rangle \sim 30$ mN m⁻¹ (refs 22, 23). The area which can be cleared by compression of the lipid for a given increase in $\langle \pi \rangle$ is obviously proportional to C . As a result it is to be expected that protein

penetration is enhanced when C is high. That this is indeed the case can be seen from Fig. 1d. It is apparent that Γ for β -casein is higher at 20 °C than at 30 °C (Γ for β -casein at the air–water interface is independent of temperature over this range) because the phase transition is prominent at 20 °C and C is much greater (Fig. 1b). We could not detect any differences in the rate of increase of Γ at the two temperatures.

The increase in π on penetration of protein (Fig. 1c) arises from the increase in packing density in the interface. The molecular packing in the mixed film can be described by a model^{9, 24} in which it is assumed that the protein penetrates and condenses the lecithin molecules to give a mosaic structure. The detailed packing of the dipalmitoyl lecithin molecules in the mixed film cannot be determined from the present data. The initial physical state of the lecithin monolayer is important through its effect on C ; for example the initially condensed monolayers formed by higher lecithin homologues⁷ give rise to a small degree of penetration with β -casein^{9, 24}. Consistent with this, the dashed lines in Fig. 1c and d extrapolate to zero $\Delta\pi$ and Γ when $A \sim 47$ Å² per molecule; at this point the phospholipid molecules are closely packed and there is no free space in the film into which protein molecules can penetrate. The presence of cholesterol which leads to the formation of condensed monolayers with lecithin¹ also reduces C and should therefore inhibit protein penetration (compare ref. 25).

We can conclude that the lateral compressibility of phospholipid monolayers and bilayers is an important parameter in determining their permeability. If *in vivo* a biological membrane is maintained so that the phospholipid bilayer contains both crystalline and liquid-crystalline regions in equilibrium then the resultant enhancement of lateral compressibility facilitates insertion of hydrophobic proteins. As a result the concentration of such proteins inside the membrane can be increased and if they are functional in transport, then the membrane permeability rises (compare ref. 3). Besides processes such as membrane transport and assembly (biogenesis), the high lateral compressibility arising when a biological membrane is maintained in the range of the gel-to-liquid crystal transition may be of fundamental importance for membrane processes in general. A phospholipid monolayer at the air–water interface is a particularly convenient model for quantitative studies of the role of lateral compressibility. The elucidation of the exact role of high lipid compressibility in membrane biology warrants a systematic investigation.

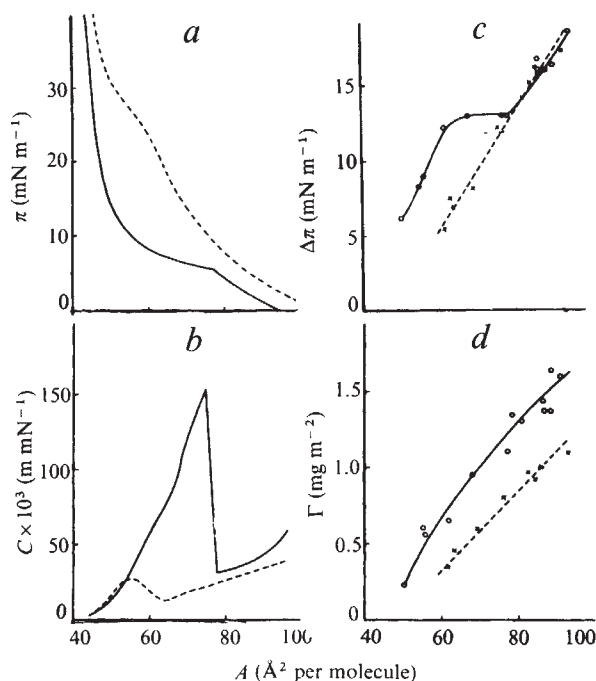
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Fig. 1 *a*, Surface pressure (π)–molecular area (A) isotherms for dipalmitoyl lecithin at the air–phosphate buffer (pH 7, $I = 0.1$) interface at 20 °C (—) and 30 °C (---). *b*, Variation of isothermal lateral compressibility of dipalmitoyl lecithin monolayers with molecular area at 20 °C (—) and 30 °C (---). *c*, The dependence of the change in surface pressure ($\Delta\pi$), after penetration of 1-¹⁴C-acetyl- β -casein, on the initial molecular area of dipalmitoyl lecithin monolayers at 20 °C (○) and at 30 °C (×). The substrate was phosphate buffer and the initial substrate concentration of β -casein was 1×10^{-4} g per 100 ml. *d*, The dependence of the final surface concentration (Γ) of 1-¹⁴C-acetyl- β -casein on the initial molecular area of dipalmitoyl lecithin monolayers at 20 °C (○) and at 30 °C (×). The other conditions were as described in *c*.



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Amino acid transport defect in glutathione-deficient sheep erythrocytes

THE tripeptide, reduced glutathione (GSH), is present in high concentration in mammalian red blood cells, where its major role is thought to be the protection of the cell against oxidative damage. Congenital red cell GSH deficiency has been reported in man, and attributed to a decreased activity of either of the two enzymes (γ glutamyl cysteine synthetase (GCS) or GSH synthetase (GSHS)) involved in GSH biosynthesis¹. Such GSH-deficient red cells have a markedly diminished lifespan, and an increased susceptibility to the haemolytic action of oxidant drugs¹.

Sheep with low concentrations of red cell GSH were first found by Smith and Osburn² and subsequent investigations have revealed at least two distinct types of congenital sheep red cell GSH deficiency^{3–5}. The first is associated with a diminished activity of the first enzyme of GSH biosynthesis (GCS) and is found in Tasmanian Merino sheep ("Merino-type")⁶. The second, found in Finnish Landrace sheep ("Finn-type"), is characterised by normal activities of both GCS and GSHS⁶, and results in a shortened red cell lifespan⁷, and an increased susceptibility to kale poisoning⁸. Significantly, in the latter, but not in the former type of deficiency, the red cells contain high concentrations of certain amino acids, particularly lysine and ornithine⁵. It is therefore possible that the low concentrations of GSH in these animals may result from a reduced amino acid permeability limiting the availability of GSH precursors, or from a direct effect of the accumulated amino acids on the enzymes of GSH biosynthesis.

To test the first possibility we have measured the permeability of normal (high GSH) and GSH-deficient (low GSH) Finn red cells to the GSH-precursor amino acids (cysteine, glutamate, glycine) and compared the results obtained with those for high and low GSH Merino cells. The data are presented in Table 1 together with values for glutamine, α -amino-*n*-butyric acid (α AB), cystine and lysine uptake by high and low GSH Finn red cells. The uptake of cysteine was rapid in Finn red cells, and sixfold greater in high GSH cells than in low GSH ones. In contrast, the uptake of cysteine by high and low GSH Merino red cells was the same, and not significantly different from that found in high GSH Finn cells. As with cysteine, glycine uptake was significantly lower in low GSH Finn red cells, although both the absolute fluxes and the difference between the GSH types were smaller. There was no significant difference in glycine permeability between high and low GSH Merino red cells. Attempts to demonstrate glutamate uptake by sheep red cells gave very low values, which were not linear with time. The impermeability of sheep red cells to glutamate is consistent with observations on human red cells⁹. Glutamine may act as a GSH precursor¹⁰ and measurements of glutamine uptake showed fluxes of the same order as for glycine, but with no significant difference between Finn GSH types.

In certain reactions (including that catalysed by GCS), α AB can substitute for cysteine^{11,12} and may therefore provide a convenient alternative to cysteine for uptake studies. The

Table 1 GSH levels and amino acid uptake rates in red cells from high and low GSH Finnish Landrace and Tasmanian Merino Sheep

	High GSH Finn 5	Low GSH Finn 5	Significance level for difference (<i>P</i>)
No. of animals			
Red cell GSH (mmol per l packed cells)	2.97 $\pm$ 0.24	1.25 $\pm$ 0.08	
Amino acid uptake (μ mol per l packed cells per h)			
Cysteine	236 $\pm$ 21	38.7 $\pm$ 4.0	<0.001
Glycine	12.7 $\pm$ 1.8	7.3 $\pm$ 0.4	<0.002
Glutamic acid	<1	<1	NS
Glutamine	11.9 $\pm$ 1.3	13.5 $\pm$ 3.1	NS
α -amino- <i>n</i> -butyric acid	656 $\pm$ 69	56.4 $\pm$ 8.6	<0.001
Cystine	8.6 $\pm$ 0.4	9.9 $\pm$ 0.5	<0.1
Lysine	19.4 $\pm$ 3.0	6.4 $\pm$ 0.8	<0.01

	High GSH Merino 6	Low GSH Merino 6	
No. of animals			
Red cell GSH (mmol per l packed cells)	3.19 $\pm$ 0.04	0.87 $\pm$ 0.04	
Amino acid uptake (μ mol per l packed cells per h)			
Cysteine	298 $\pm$ 12	242 $\pm$ 2	NS
Glycine	10.4 $\pm$ 0.8	11.7 $\pm$ 0.6	NS

Data presented as mean $\pm$ s.e.m.

NS, not significant.

Washed sheep red cells were incubated at 10% haematocrit in a medium containing (mM): NaCl 135, KCl 5, Tris-HCl (pH 7.1 at 37 °C) 15, MgCl₂ 3.1, EDTA 0.1, amino acid 0.2 (containing ¹⁴C-amino acid at 0.2 μ Ci ml⁻¹). Since cysteine oxidises rapidly in solution at neutral pH, cysteine uptake was measured in the presence of 10 mM dithiothreitol. Control experiments showed no effect of 10 mM dithiothreitol on α AB influx or efflux. At fixed time intervals (10–30 min for Gly, α AB, Lys, Cys/2; 30–90 min for Cys, Gln, Glu), aliquots (1 ml) were taken and the cells washed four times in ten volumes of a medium containing (mM): MgCl₂ 106, Tris-HCl (pH 7.4 at 4 °C) 10, by centrifugation (10 s, 10,000g) in an Eppendorf 3200 microcentrifuge. Finally, the packed cells were lysed in 0.5% Triton X-100 in water (0.5 ml), 33% trichloroacetic acid added (0.5 ml) and the precipitate removed by centrifugation. An aliquot of supernatant (0.9 ml) was placed into Bray's solution¹⁴ (10 ml) and counted in a β scintillation spectrometer with quench correction.

uptake of this amino acid by Finn red cells was therefore measured, and found to be rapid, with the flux in high GSH cells some tenfold greater than that encountered in low GSH cells. In contrast to the situation with cysteine and α AB, cystine (the oxidised form of cysteine) showed a slow rate of uptake, with no significant difference between high and low GSH Finn cells. The uptake of lysine, which is found at concentrations of up to 20 mmol per litre packed cells in low GSH Finn red cells was measured in high and low GSH Finn cells. High GSH cells showed a threefold greater lysine permeability than low GSH cells.

The above results show that differences in amino acid transport, particularly for cysteine, α AB and lysine exist between high and low GSH Finn red cells. In contrast to glutamate and glycine, the concentration of cysteine in sheep red cells is very low (13 μ mol per litre packed cells)¹³. Thus a diminished cysteine availability may be responsible for the low concentrations of GSH in low GSH Finns.

The reduced amino acid transport in low GSH Finn red cells is not a direct consequence of a low intracellular GSH concentration, since low GSH Merino red cells show normal amino acid uptake values. Further experiments were designed to determine whether the diminished amino acid uptake by low GSH Finn red cells resulted from a membrane transport defect or whether it was a function of the high lysine and ornithine content of these cells. Uptake of α AB (0.2 mM) by high GSH Finn red cells was unaffected by the presence of extracellular lysine or ornithine (10 mM). Similarly, α AB efflux from the same cells (preloaded by incubation in the

presence of 1 mM α AB for 2 h at 37 °C) was unaffected by extracellular lysine or ornithine at a concentration of 5 mM. In contrast, 10 mM cysteine inhibited α AB uptake by 50% and stimulated α AB efflux by 30% under equivalent conditions. Finally, high GSH Finn red cells were preloaded with lysine by incubation in the presence of 10 mM lysine for 20 h at 20 °C. Uptake of α AB (0.2 mM) by these cells (intracellular lysine concentration 3.0 mmol per litre packed cells) was identical to that of control high GSH cells which had been incubated for the same length of time in the absence of lysine (intracellular lysine concentration 0.1 mmol per litre packed cells).

We therefore conclude that the diminished amino acid uptake of low GSH Finn red cells represents a membrane transport defect which is not a consequence of the low GSH or elevated lysine and ornithine concentrations in these cells. It further seems likely that diminished availability of cysteine is responsible for the low concentrations of red cell GSH in these animals, and that the accumulation of lysine and ornithine is a further reflection of reduced amino acid transport.

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New type of infectious complex of *E. coli* RNA phage

PREPARATIONS of the three components of RNA phage—RNA, coat protein and maturation protein (A protein)—have been used in several attempts to reconstitute the particles *in vitro*¹⁻⁷. Particles recovered so far have been similar to authentic particles with respect to S value, morphology and other properties. We have now reconstituted a complex, by mixing RNA and A protein only, which can infect *Escherichia coli* regardless of its small S value, 28S.

Preparation of phage RNA and A protein, and conditions for reconstitution are described in the legends to Table 1 and Fig. 1. Polyacrylamide gel electrophoresis showed that A protein was not contaminated by coat protein. Infectivity of MS2 RNA-MS2 A protein complex, measured as described in the legend to Table 1, was detected only when RNA and A protein were mixed, and it seemed to increase when the mixture was dialysed. The greatest efficiency of infectivity observed was approximately 1.4×10^{-5} plaque-forming units (PFUs) per RNA strand (after dialysis). Sucrose gradient centrifugation of the mixture gave peaks of absorbance and infectivity corresponding to those of RNA (Fig. 1a and b). This suggests that the major component of the reconstituted complex is a single molecule of RNA, and that one or a few molecules of A protein play some roles in infecting intact host cells.

By assaying infectivity of the complex on *E. coli* K12 Hfr Q13, F⁺ A/ λ , F⁻ W3110 and C15 (a derivative of A/ λ which is resistant to MS2 and GA phages), we found that the host range

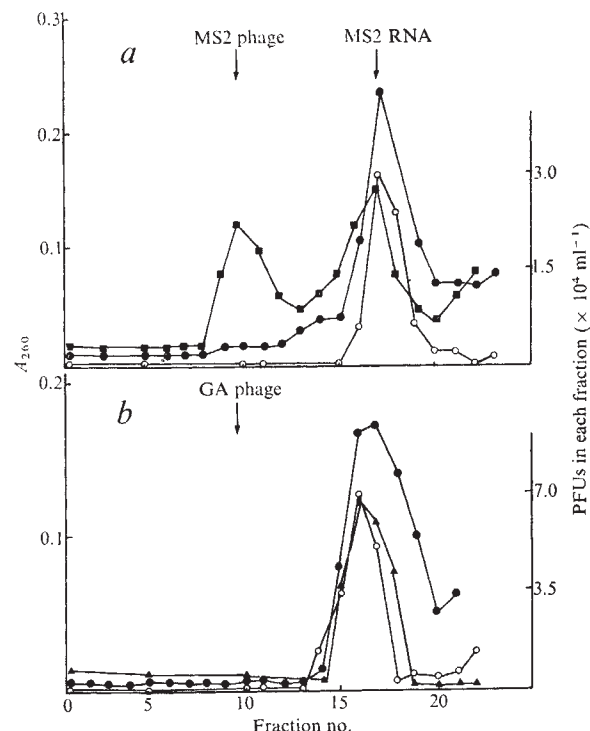


Fig. 1 Formation of RNA-A protein complex. Phage RNA and phage protein were isolated from purified phage by the SDS-phenol method¹². The isolated RNA was purified further by sucrose gradient centrifugation and ethanol precipitation. The phage mixed protein (coat protein and A protein) was recovered from phenol layer by the method of Hung *et al.*⁴, and dissolved in 5 M guanidine-HCl, plus 0.05 M mercaptoethanol in 0.1 M Tris-HCl buffer (pH 7.2). A protein was isolated from MS2 phage and GA phage by the method of Osborn *et al.*¹³. A protein was dissolved in 5 M guanidine-HCl, plus 0.05 M mercaptoethanol in 0.1 M Tris-HCl buffer (pH 7.2). A 1-ml sample of reaction mixture contained the following components; 5 μ g of MS2 (a) or GA (b) A protein, 100 μ g of MS2 (a) or GA (b) RNA (dissolved in 0.01 M Tris-HCl (pH 7.2), 0.005 M $MgCl_2$), 5 mmol of guanidine-HCl, and 50 μ l of mercaptoethanol in 0.1 M Tris-HCl buffer (pH 7.2), and 200 μ g of phage mixed protein if used. After dialysis as in Table 1, the reaction mixture was centrifuged in a 5-20% (w/v) linear sucrose density gradient at 1 °C for 4.7 h (25,000 r.p.m.) in a Spinco SW 27 rotor. Fractions were collected from the bottom of the tube and analysed for infectivity and absorbance at 260 nm. a: $\blacksquare$, MS2 RNA+MS2 A protein + MS2 mixed protein; $\bullet$, absorbance of MS2 RNA-MS2 A protein complex; $\circ$, infectivity of MS2 RNA-MS2 A protein complex; b: $\bullet$, absorbance of GA RNA-GA A protein; $\circ$, infectivity of GA RNA-GA A protein complex; $\blacktriangle$, absorbance of GA RNA.

of the complex was identical to that of the phage from which RNA and A protein were prepared.

The sensitivity of the complex (MS2 RNA-MS2 A protein) to anti-MS2 serum was tested and, unlike intact MS2 phage

Table 1 Infectivity of MS2 RNA-MS2 A protein complex

Assay mixture	PFU/RNA (μ g)
RNA (before and after dialysis)	$< 10^2$
A protein (before and after dialysis)	$< 10^2$
RNA + A protein (before dialysis)	6×10^4
RNA + A protein (after dialysis)	2.2×10^5

A 1-ml sample of reaction mixture contained the following components: 5 μ g of MS2 A protein, 50 μ g of MS2 RNA (dissolved in 0.01 M Tris-HCl (pH 7.2), 0.005 M $MgCl_2$), 5 mmol guanidine HCl, and 50 μ l of mercaptoethanol in 0.1 M Tris-HCl buffer (pH 7.2). If necessary the mixture was dialysed against Tris-acetate buffer (0.1 M Tris-HCl (pH 7.2), 0.05 M KCl and 0.02 magnesium acetate) in an ice bath for 18 h. PFUs in the reaction mixture were assayed on K12 F⁺ A/ λ .

Table 2 Sensitivity of MS2 RNA-MS2 A protein complex to anti-MS2 serum

	Control (PFU ml ⁻¹)	+Anti-MS2 serum (PFU ml ⁻¹)
Complex	3 × 10 ⁵	5 × 10 ⁵
MS2 phage	1.1 × 10 ⁷	4.1 × 10 ⁴

A 0.9-ml sample of a solution of MS2 RNA-MS2 A protein complex or MS2 phage suspension was mixed with 0.1 ml of anti-MS2 serum (K value = 5) and incubated at 37 °C for 10 min. Remaining PFUs were assayed on K12 F⁺ A/λ. In control, the complex or the phage was similarly treated in the absence of the serum.

particles, the complex was resistant to the serum (Table 2).

The complex was much more sensitive to RNase, trypsin and Pronase than were intact phage particles (Table 3). The extreme sensitivity to RNase suggests that a naked RNA is a component of the complex.

Analysis of the physical nature of the complex indicated that it was rather unstable. Infectivity decreased to one-tenth after storage for 24 h at 4 °C. Also heat pretreatment of the RNA destroyed infectivity. Heating at 70 °C for 10 min in TM buffer (0.005 M MgCl₂ in 0.01 M Tris-HCl, pH 7.2) decreased the activity to one-eighth to one-twentieth, and at 100 °C to 10⁻⁴ or less.

We conclude that a complex infectious to intact *E. coli* cell

Table 3 RNase, trypsin and Pronase-sensitivity of MS2 RNA-MS2 A protein complex

Enzyme (μg ml ⁻¹)	Survivors (PFU ml ⁻¹)	
	Complex	MS2 phage
RNase (μg ml ⁻¹)		
10 ⁻¹	< 10 ²	5.4 × 10 ⁵
10 ⁻²	< 10 ²	NT
10 ⁻³	< 10 ²	NT
10 ⁻⁴	< 10 ²	NT
—	1.4 × 10 ⁴	6 × 10 ⁸
Trypsin (μg ml ⁻¹)		
10	< 10 ²	6 × 10 ⁸
1	< 10 ²	5.5 × 10 ⁸
0.1	8 × 10 ³	NT
0.01	1.6 × 10 ³	NT
—	8 × 10 ³	6 × 10 ⁸
Pronase (μg ml ⁻¹)		
50	< 10 ²	6 × 10 ⁸
25	< 10 ²	NT
10	< 10 ²	6 × 10 ⁸
1	9 × 10 ³	NT
—	8 × 10 ³	6 × 10 ⁸

A sample of 0.1 ml of a solution of MS2 RNA-MS2 A protein complex was mixed with 0.9 ml of enzyme solution (Pronase-P, Kaken Chemical Co.; Trypsin-Type 1, Sigma; RNase-Type 1-A, Sigma) dissolved in TM (0.01 M Tris-HCl (pH 7.2), 0.005 M MgCl₂), and treated for 10 min at 37 °C.

NT, not tested.

was formed by mixing RNA and A protein of RNA phage. Although the structure of the complex is unknown, our data suggest that it was composed of a single naked RNA molecule and one or a few A protein molecules. Such a complex is probably a minimum infectious unit of RNA phage, and coat protein is dispensable in this respect. The complex might have some significance for consideration of the origin and evolution of viruses. An RNA-A protein complex has been reported in infected cells and so has penetration of the complex into host cells on infection by phage⁸⁻¹¹. These complexes might be identical to our infectious complex reconstituted *in vitro*, although infectivity was not reported in the other cases.

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Retention of tumour-inducing capacity by adenovirus DNA after cleavage by restriction endonucleases

We have previously reported that fragments of Simian adenovirus SA7 DNA produced by physical breakage are oncogenic¹. These fragments had an average molecular weight corresponding to half-molecules (11.4 × 10⁶). After separation of the heavy and light molecular halves by density gradient centrifugation, we demonstrated that both half-molecule preparations retain the ability to initiate tumours in newborn hamsters. We suggested that this may be caused by the presence of one or more "oncogenic regions" in both molecular halves, or by random breaks near the centre of the DNA which could result in a single small oncogenic region, residing near the centre and occurring with equal probability in either the heavy or light half-molecule preparation. To clarify these results we have studied the effect of several restriction endonucleases on the oncogenic activity of SA7 DNA. These enzymes produce defined fragments of the SA7 genome rather than the heterogeneous populations produced by physical shear.

DNA was extracted using a phenol method from virus that had been banded twice in CsCl gradients². Restriction endonucleases *EcoRI* and *EcoRII* were purified from *Escherichia coli* strain R204 and R15, respectively (Dr D. Jackson). Endonuclease *Hind* was purified⁴ from *Haemophilus influenzae* (strain Rd, Dr H. Smith). Endonuclease *Hga* was obtained from *H. gallinarum* (ATCC 14385) using a modification of the method of Takanani and Kojo⁵. Endonucleases *HpaI* and *HpaII* were purified⁶ from *H. parainfluenzae* (Dr J. Setlow).

The number of fragments produced by the various enzymes was found to vary from two to more than twenty (Table 1). *EcoRI* was found to make a single break in SA7 DNA. The result of analytical ultracentrifuge analysis (Fig. 1) of DNA hydrolysed by *EcoRI* revealed heavy and light fragments with buoyant densities corresponding to 61.1 and 53.9% guanine + cytosine (G + C) respectively, compared with intact SA7 DNA of 58% G + C. Molecular weights for the heavy and light fragments were calculated⁷ and corresponded to 10.7 and 11.6 × 10⁶ dalton, respectively. The alkaline sedimentation coefficient for the mixed fragments (*S*_{20,w}⁰ = 26.2) corresponded to a single strand molecular weight of 5.5 × 10⁶. These fragments are very similar to the heavy and light half-molecule fragments produced by shear, except they represent homogeneous populations resulting from breakage of the DNA at a unique site.

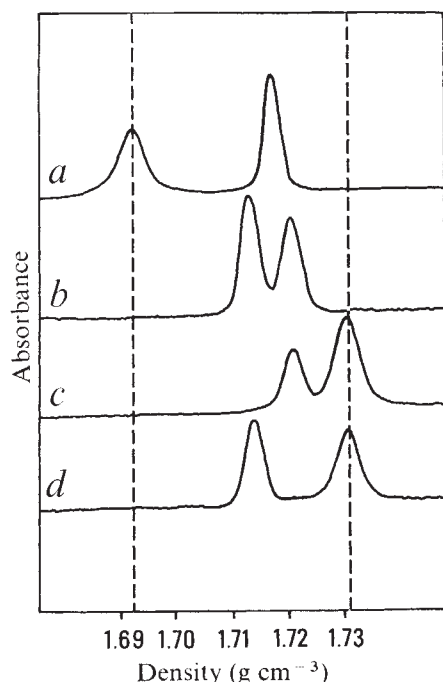


Fig. 1 Model E ultracentrifuge scanner tracings of DNA preparations in CsCl-buoyant density experiments. Two marker DNAs are shown: *C. perfringens* ($\rho = 1.692$) and *M. lysodeikticus* ($\rho = 1.731$). The *EcoRI* fragments were separated on preparative NaI-EtBr gradients in a type 40.1 rotor (Beckman) by centrifugation for 100–200 h at 36,000 r.p.m. The visible DNA bands were recovered and NaI was removed by dialysis. Ethidium bromide was extracted from the dialysed DNA with isopropanol (three times). a, Intact SA7 DNA, $\rho = 1.717$; b, SA7 DNA cleaved by *EcoRI*; c, *EcoRI* heavy fragment, $\rho = 1.720$; d, *EcoRI* light fragment, $\rho = 1.713$.

The mixture of fragments produced by each restriction enzyme was tested for oncogenicity by subcutaneous injection into newborn hamsters within 24 h of birth. DNA (5 μ g) was administered to each animal. That cleaved by *EcoRI* or *HpaI* retained the ability to initiate tumours (Table 1), whereas all the other preparations failed to yield tumours in the conditions tested. Contamination by intact DNA could not account for tumour induction by either *HpaI* or *EcoRI* fragments as gel electrophoresis data preclude contamination by more than 1%.

Table 1 Tumour induction by SA7 DNA fragments

Enzyme	No. of fragments	DNA/animal	Tumour incidence	Length of test (d)
<i>Hind</i>	20–25*	5 μ g	0/34	365
<i>Hga</i>	20–25*	5 μ g	0/56	85
<i>HpaI</i>	7	5 μ g	17/47	169
<i>HpaII</i>	20–25*	5 μ g	0/42	169
<i>EcoRI</i>	2	5 μ g	7/35	282
<i>EcoRI</i> —Heavy		2.5 μ g	4/59	120
<i>EcoRI</i> —Light		2.5 μ g	0/77	120

SA7 DNA was digested with each enzyme for 1 h followed by addition of fresh enzyme and further incubation for 1–2 h. In each case, the digestion products were examined using agarose or acrylamide-agarose gel electrophoresis to be certain that limit digests were obtained and that no detectable intact DNA remained. (Approximately 0.01 μ g SA7 DNA can be detected using EtBr staining. Since 3–6 μ g DNA was normally electrophoresed, intact DNA would represent at most less than 1% of the total.) At the end of the digestion period, each preparation was deproteinised with phenol and dialysed against SSC (0.15 M NaCl, 0.15 M sodium citrate, pH 7.0).

* The number of fragments was estimated using electrophoretic separation 2.4% acrylamide–0.5% agarose gels. More than twenty, but less than twenty-five bands could be distinguished for *Hind*, *Hga*, and *HpaII*.

This amount of DNA per animal (0.05 μ g) is insufficient to induce tumours at a frequency greater than 1% (J.P.B., N.M., and L.H., unpublished). When the heavy and light fragments produced by *EcoRI* were tested individually at a 2.5- μ g dose, only the heavy fragments were oncogenic.

This result demonstrates that the information required for tumour induction is probably unique and occurs only in the heavy half of the SA7 genome. Thus, of the two possible explanations suggested for our previous experimental results with fragments produced by shear¹, the second alternative is more likely. The existence of a single small oncogenic region of DNA near the centre of the SA7 molecule could therefore be found only in the heavy half-molecule after cleavage at a unique site by *EcoRI*, but may be found with roughly equal probability in both half-molecule preparations generated by random breaks resulting from shear. (This is based on the fact that a break in the DNA molecule on the left or right side of such an oncogenic region would result in the activity being found in the light or heavy half-molecule respectively, whereas a break in the region itself may result in loss of activity.) If this explanation is correct, we would expect to find the oncogenic activity near the central terminus of the *EcoRI* heavy half-molecule fragment. We hope to resolve this in the near future, since we know that *HpaI* produces six breaks in SA7 DNA, but only one of these is in the heavy *EcoRI* fragment of the molecule. We are currently preparing and injecting these fragments to determine which *HpaI* piece (or pieces) is responsible for the production of tumours by the *HpaI* digest.

Several authors have presented evidence that the transforming genes of human adenovirus type 2 may be near the left end of the Ad2 DNA molecule. Gallimore *et al.*⁸ have analysed the DNA of rat cells transformed by Ad2 and found that some lines contain as little as 14% of the virus genome and that this region corresponds to the left end of the molecule. Additionally, Graham *et al.*⁹ presented evidence that half-molecule fragments of human adenovirus type 5 DNA produced by shearing the DNA would transform primary rat cells in culture. The transforming activity was found in the left or heavy (high G+C) half-molecule fraction. They presented further evidence which suggested that the activity could be found in fragments as small as 10^6 dalton and may be localised in a region between 1 and 6% from the left end of the molecule. Our results differ in the following ways. First, we measured tumour induction *in vivo* rather than transformation *in vitro*. Although we were able to obtain transformation *in vitro* with Ad5 DNA, we have not been able to induce tumours with either intact Ad5 DNA or *EcoRI* fragments (J.P.B., N.M., and L.H., unpublished). Second, we have not been able to induce tumours with SA7 fragments smaller than 5×10^6 dalton using either physically sheared material (ref. 1 and J.P.B., N.M., and L.H., unpublished) or restriction enzyme fragments.

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Dark-repair of ultraviolet-induced pyrimidine dimers in the DNA of wild carrot protoplasts

GREEN plants receive greater exposure to solar ultraviolet radiation and their germ cells (pollen) have a significantly greater potential for acquiring ultraviolet-induced genetic damage than do those of most animals¹. Even though cells of the higher plants can photoreactivate ultraviolet damage (refs 2-5, and my unpublished data), the absence of dark-repair capability could be a significant disadvantage since some types of excisable ultraviolet-induced DNA damage are not photoreactivable⁶. The capability for dark repair would become even more important if there were an increase in the fluence of solar ultraviolet reaching the Earth's surface⁷. Attempts to demonstrate excision of pyrimidine dimers in cells of *Nicotiana*, *Haplopappus*⁴, *Ginkgo*⁵ and *Chlamydomonas*⁸ have all given negative results. These data, taken with other assays for excision repair activity (unscheduled DNA synthesis⁹; repair replication¹⁰⁻¹²) have been interpreted as indicating a general absence of such capability in plants¹². I have now found, however, that in cultured wild carrot cells, pyrimidine dimers can be excised in the dark and that the extent of dimer excision depends on the initial number of dimers induced by the ultraviolet dose. After ultraviolet fluences of about 10 J m⁻², most of the dimers are excised within 24 h; but at fluences up to 30 J m⁻², about 25% remain unexcised in DNA. At fluences above 100 J m⁻², dimer excision is almost completely eliminated, perhaps due to secondary effects of these very high ultraviolet doses.

The results of dimer analyses on wild carrot DNA are presented in Fig. 1 and Table 1. The yield of dimers immediately after irradiation was linear for ultraviolet fluences of 0-450 J m⁻² and was equal to 0.002% radioactivity in thymine-containing dimers per radioactivity in thymine (X [T]/T) per J m⁻² dose. Thus the efficiency of thymine dimer formation was 35-40% that observed in bacteria¹³ and mammalian cells¹⁹. This difference may be attributable to the shielding effect of cytoplasmic organelles in wild carrot protoplasts.

The excision data fit a curve showing 100% dimer removal at lower ultraviolet doses. Essentially complete excision of up to about 0.02% X [T]/T occurred within 24 h, as reported in human cells for dimer levels up to about 0.01% X [T]/T (ref. 20). My data for excision in wild carrot protoplasts are minimum values compared with those from work on animal cells, since in the latter case any dead cells are eliminated from the assay because they do not adhere to the surface of the culture dish¹⁹.

Excision was almost completely eliminated at doses above 100 J m⁻², although a few dimers were excised even at the highest doses (Table 1). The 2% excision observed after 150-450 J m⁻² of ultraviolet amounted to about 10⁶ excised dimers per cell.

Alkaline sucrose gradient analysis revealed about 0.2 single-strand gaps per 10⁸ daltons of DNA appearing immediately after 35 J m⁻² 254-nm irradiation of wild carrot protoplasts (my unpublished data). This implies about 10⁴ dimer-specific endonuclease molecules per cell, if the number of gaps corresponds to the average number of repair molecules. Similar data have been obtained for mammalian cells²³. A fluence of 42 J m⁻² induced approximately 8.4 × 10⁵ pyrimidine dimers per cell. (These data are based on a 2C nuclear DNA content of 2.1 × 10⁻¹² g per cell²⁴ and on an approximation of the relative frequencies of C[C], C[T] and T[T] dimers^{9,18}.) Since 58 ± 15% of these lesions were excised in 24 h, the average rate of excision was 20,000-30,000 dimers h⁻¹, comparable with that estimated for excision repair in mammalian cells^{20,23}.

At 30-60 J m⁻² maximum excision (>70%) was observed after 24 or 40 h dark incubation. Excision seemed to stop short of removal of all dimers, however, with no additional excision

in the longer incubation. All the dimers induced by 42 J m⁻² could be photoreactivated (Table 1), so that this apparent limit for excision was not an artefact of the dimer assay procedure. Clayton *et al.*²⁵ have shown that dimers are seemingly not excised from mitochondrial DNA in three mammalian cel

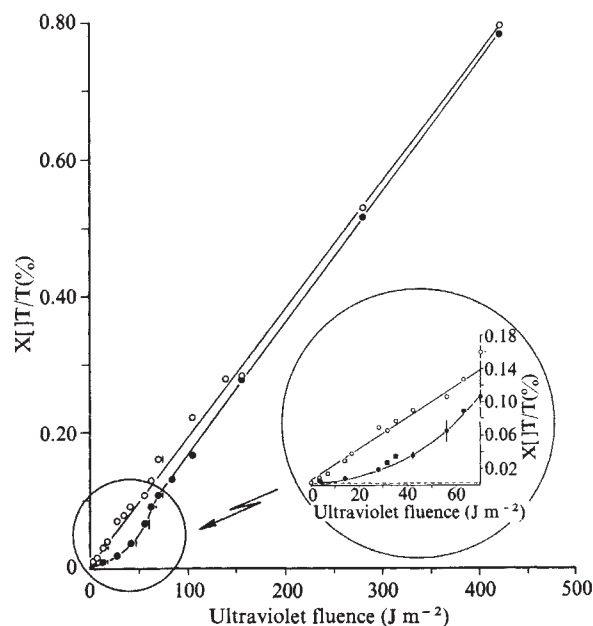


Fig. 1 Amount of thymine dimers as a function of ultraviolet fluence immediately after irradiation (○) and after ultraviolet followed by 24 h (●) or 40 h (■) dark incubation.

$$X [T]/T = \frac{\text{radioactivity in thymine-containing dimers}}{\text{radioactivity in thymine}} \times 100.$$

Standard error bars shown around the mean of three to nine separate samples. Dashed line indicates background dimer level in unirradiated samples. The source and culture of wild carrot cells (*Daucus carota* L.) have been described¹³. Cellular DNA was labelled for 20-24 h by incorporation of methyl-³H-thymidine (17 Ci mmol⁻¹, Amersham/Searle) supplied at 5 μCi ml⁻¹ in the culture medium. Fluorodeoxyuridine at 10⁻⁵ M was also included to inhibit the degradation of thymidine and to enhance the specific labelling of DNA (refs 21 and 22 and my unpublished data). Cell walls were digested and protoplasts isolated and counted as before¹³. The specific radioactivity (acid-insoluble) was 4 ± 1 c.p.m.⁻¹ per protoplast on average. Protoplasts (1.0 × 10⁵-1.5 × 10⁵ ml⁻¹) were irradiated as a disperse monolayer in Petri dishes through about a 2-mm depth of protoplast isolation medium¹³ with agitation at room temperature. The 2-mm thickness of medium transmits about 50% of the radiation at 254 nm; therefore a correction factor¹⁴ of 0.70 was applied to the measured ultraviolet fluences. Ultraviolet was delivered by a germicidal lamp (primarily 254 nm) at a fluence rate of 0.71 J m⁻² s⁻¹ as measured with a Jagger meter¹⁵. The use of isolated protoplasts allowed reproducible, uniform ultraviolet exposures and quantitative induction of pyrimidine dimers. During and after irradiation the protoplasts were handled only under non-photoreactivating illumination. Thymine-containing dimers in samples of isolated¹⁶ DNA were analysed by two-dimensional paper chromatography¹⁷. For the lowest ultraviolet doses, two to three times more total radioactivity (in hydrolysed DNA) was applied to each chromatogram to allow recovery of a significant level of counts in the dimer region.

lines that can excise dimers induced in nuclear DNA. No dimer excision was detected in *Chlamydomonas* chloroplast DNA⁸. If this were the case in my wild carrot protoplasts, about 20% (the approximate proportion of organellar DNA in these cells) of the dimers would have remained after maximum excision. This, in fact, is the approximate limit reached after ultraviolet fluences up to about 30 J m⁻² followed by 1 d dark incubation. Experiments are now in progress to evaluate the

Table 1 Representative dimer analyses in wild carrot protoplasts

Ultraviolet fluence (J m ⁻²)	Dark incubation (h)	Net c.p.m.		X[TT/T] (%)	Excision* (%)
		Dimer region	Thymine region		
14	0	277	1,560,000	0.018	
14	24	40	1,550,000	0.002	100
42	0	459	538,000	0.085	
42	24	131	338,000	0.039	56
42	†	2	180,000	0.001	(100)†
105	0	664	297,000	0.224	
105	24	580	352,000	0.165	27
280	0	1431	271,000	0.529	
280	24	724	140,000	0.516	2.5
420	0	1870	234,000	0.797	
420	24	1570	200,000	0.784	1.6

*Background (0.0034 ± 0.0007% X[TT/T; n = 5) subtracted.

†Complete photoreactivation of pyrimidine dimers by 24 h incubation under about 300 foot-candles cool-white fluorescent light (25 °C).

possibility that pyrimidine dimers are not excised from organellar DNA in these cells.

Although it is clear that pyrimidine dimers can be excised from the DNA of isolated protoplasts of cultured wild carrot cells, the distribution of this capacity among higher plants has yet to be determined. Wild carrot protoplasts show a greatly reduced level of dimer excision when there are many dimers present (that is, initial levels greater than about 0.1% X[TT/T). This may explain previous failures to detect excision in tobacco (0.40% X[TT/T initial dimer level)⁴, *Haplopappus* (1.12%)⁴, *Ginkgo* (2.22%)⁵ and *Chlamydomonas* nuclear (equivalent to 0.33–0.66%)⁸ and chloroplast DNAs (equivalent to 2.31%)⁸. Other reports indicating an absence of excision repair activity in higher plant cells^{9–12} must now be re-evaluated.

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Note added in proof: Soifer and Cieminis have reported dimer excision in whole grass pea, *Lathyrus sativa* L.) embryos²⁶.

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Progressive decrease in protein synthesis accuracy induced by streptomycin in *Escherichia coli*

The passage of genetic information from gene to polypeptide does not proceed with perfect fidelity^{1–3}, but it is not known what short and long-term cellular responses may be induced by decreases in the accuracy of protein synthesis. Errors in gene expression may tend to increase their own rate of production⁴, and thus could constitute a limiting instability of cellular life. The synthesis of inaccurate RNA, RNA polymerases, tRNAs, tRNA modifying enzymes, tRNA charging enzymes and ribosomal proteins are potential pathways for the perpetuation or amplification of errors. Such errors may be inducible *in vivo* by external chemical agents and may constitute an indirect pathway for mutagenesis^{5–8}. An enhanced rate of such errors may be the mechanism by which certain deleterious mutations exert their

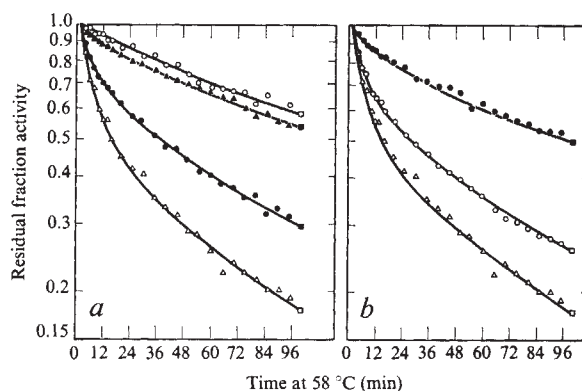


Fig. 1 Streptomycin-induced, time-dependent changes in the thermal stability of β -galactosidase. A culture of a streptomycin-sensitive, lac permeaseless derivative of the K12 strain W1485 (called IH79) was grown at 37 °C in enriched medium (Luria broth and phosphate-buffered minimal A, as described in Miller¹⁰, 1:1) to the mid-log phase and maintained there during the experiment by dilution into prewarmed medium. Streptomycin (2 μ g ml⁻¹) was added at generation time 0 in this experiment, and sample subcultures were withdrawn at the generation time indicated. Each sample was induced by the addition of 1.0 mM isopropyl- β -D-thiogalactopyranoside for 30 min, thoroughly washed on a Millipore filter and resuspended in buffer A. Reducing buffer¹¹ (0.2 ml ml⁻¹) was added to each sample, which was then lysed with chloroform (0.02 ml ml⁻¹). After 20 min of agitation the samples were centrifuged at 4,000 r.p.m. for 20 min, and the remaining chloroform was removed from the supernatant by evaporation with agitation at 37 °C. (Samples lysed in a pressure cell gave identical kinetics of β -gal thermal denaturation.) Portions (1 ml) of each sample were incubated in plastic tubes at 58 °C (stable to within ± 0.05 °C) for various times determined by varying the starting time. The incubation was stopped by agitating all the tubes in 19 °C water for 1 min. The tubes were then allowed to stand at 37 °C for 90 min before assay for β -gal activity by hydrolysis of orthonitrophenol- β -D-galactopyranoside at 37 °C (ref. 12). The residual fraction of enzymatic activity is plotted as a function of the time at 58 °C. The zero and 100-min points are each the mean of five samples. The generation time for each curve is the mid-point of the sample induction period calculated from the history of absorbance readings (650 nm) taken from the main culture. Repeated runs with improved resolution showed that, with this procedure, the decay kinetics of β -gal from untreated cultures is first order to within 2–3% for 200 min after the first 5 min. *a*, Curves for samples out to 7.5 generation, *b*, the two subsequent samples with the 7.5 generation curve repeated. Samples taken at the indicated number of generations (doublings): *a*, $\circ$, 0; $\blacktriangle$, 1; $\bullet$, 4.1; $\triangle$, 7.5. *b*, $\triangle$, 7.5; $\circ$, 11; $\bullet$, 16.

effects. Here we report the results of experiments with *Escherichia coli* designed to detect evidence of self amplification of errors in protein synthesis. If such error feedback is quantitatively significant, a slight increase in the pre-existing error rate should be followed by a progressive decrease in the accuracy of protein synthesis. While this process could lead to a new stable level and thus need not precipitate a catastrophic breakdown, some late effects should be observable if the mechanisms are at all as hypothesised.

We have manipulated the error rate in protein synthesis by exposing the cells to low levels of streptomycin, an aminoglycoside antibiotic which increases the rate of ribosome-mediated misincorporation of amino acids⁹. The fidelity of protein synthesis in a culture at a given time was assayed by measuring the thermal decay kinetics of the enzyme β -galactosidase (β -gal) produced by a freshly divided and induced subculture. The thousandfold inducibility of this enzyme permits a reasonably sharp definition of the time at which the accuracy of protein synthesis is measured.

In the basic experiment in this series, a culture was sampled just before and at various times after addition of streptomycin at concentrations in the range 0.5 to 5 $\mu\text{g ml}^{-1}$. Typical results are shown in Fig. 1. Before addition of streptomycin the β -gal thermal denaturation curve had reasonably first-order characteristics, indicating a correspondingly homogeneous enzyme population. After addition of the drug a progressively increasing fraction of the newly synthesised β -galactosidase molecules showed enhanced thermolability.

Simultaneously with these changes in the β -gal thermolability pattern, the cultures themselves showed a delayed, progressively decreasing growth rate and a progressive increase in the production of multinucleate, filamentous cells. Recovery of the culture, as measured by thermal decay of β -gal, (Fig. 1b) was also accompanied by increased growth rate and disappearance of elongated cells. These observations support the idea that the thermal decay assay detects a specific consequence of a general cell-wide effect.

A series of experiments similar to that depicted in Fig. 1 lead us to several additional observations and tentative conclusions concerning this effect. (1) Both the maximum extent and speed of development of the effect showed nonlinear, direct dependence on the concentration of streptomycin. (2) Maximum extent of the effect was correlated with the speed of its development. The smaller the effect, the longer it takes to develop; it was maximal in 8 to 10 generations for high drug concentration (2–5 $\mu\text{g ml}^{-1}$) and in 14 to 17 generations for low concentration (about 1 $\mu\text{g ml}^{-1}$). These observations seem inconsistent with the idea that the simple replacement of some cell component by its post-treatment equivalent (without feedback) is responsible for the progressive nature of the effect. (3) The effect was sensitive to the growth medium and growth history of the culture. (4) Within a fourfold range of growth rates (produced by varying the growth medium), generation time and not the actual time seemed to determine the kinetics of development. (5) With treatments mild enough to allow growth for a few generations after addition of streptomycin, the cultures ultimately recovered. Recovery to normal growth rate and cell morphology was always correlated with decreased levels of thermolabile β -gal. We have, however, never seen complete recovery in as many as 25 generations after addition of the drug.

We do not know whether recovery was due to adaptation within the cells or to selection of some subtly resistant variants. If a favoured phenotype was being selected, however, it must have been present initially at a concentration of about 1 in 10^9 . The recovered populations are not resistant to streptomycin at levels of 20 $\mu\text{g ml}^{-1}$ or higher; hence, classical streptomycin-resistant alleles of the *strA* locus are not involved.

Since we observed increased heterogeneity of the thermal decay rates for β -gal, it seems unlikely that the change was due to some other factor produced by the treated cells, which persisted through our washing, resuspension and lysis proce-

dures and enhanced the thermolability of the enzyme. To check this possibility, however, we mixed lysates from induced but untreated cells in various ratios with extracts from late, drug-treated cultures that contained only basal levels of β -gal. The thermolability of the enzyme was unaffected even when the extract from treated cells was in fivefold excess and when parallel induced samples of the treated cultures showed the expected enhanced thermolability.

A convincing test of the same question comes from an experiment designed to show whether the increased thermolability is due to modification of the enzyme after synthesis. Since enzyme synthesised before addition of streptomycin remained unaffected (Fig. 2), we tentatively attribute the observed alteration of the protein to faulty protein synthesis.

Another experiment showed that the growth of late streptomycin-treated cultures was much more sensitive to temperature than that of untreated cultures (Fig. 3), implying that thermolabile, but otherwise functional, protein required for growth is being produced in the treated cells.

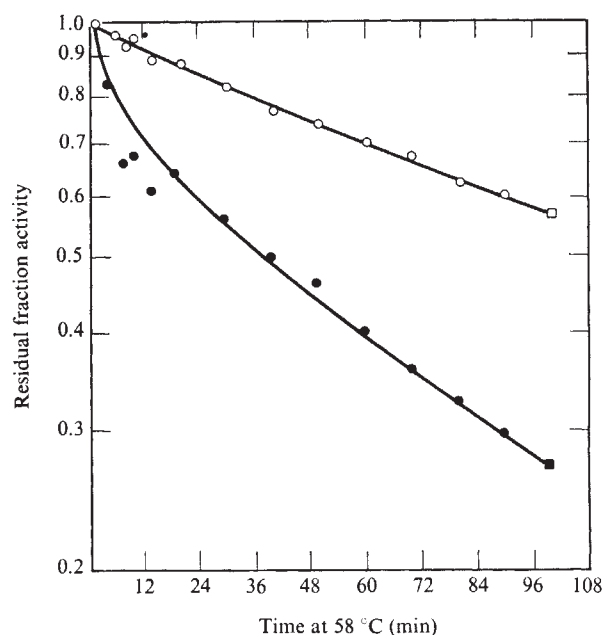


Fig. 2 Thermolability seems to be conferred at the time of protein synthesis. Induced cells grown overnight to produce maximum levels of β -gal were deinduced by washing on a Millipore filter, and resuspended in medium containing streptomycin (2 $\mu\text{g ml}^{-1}$). A control sample was taken immediately (but not further induced) and the culture was allowed to grow for about 5.5 generations. It was then divided into two samples (○ and ●), one of which (●) was induced for 30 min. The procedures and conditions were otherwise as described in Fig. 1. This figure shows the thermal decay curves resulting from these sample cultures. The decay kinetics of the control sample were characteristic of untreated cultures and indistinguishable from the zero generation curve in Fig. 1 and from curve ○ in this figure.

If we were observing a progressive breakdown in the accuracy of protein synthesis, it could have been due to causes other than error feedback: a slow increase in the uptake of streptomycin, possibly the result of a feedback cycle directly affecting permeation, or of some process involving the metabolism or binding of the drug. Indirect evidence that such effects are not implicated came from our examination of the immediate effect of high doses of streptomycin on the production of thermolabile β -gal (data not shown). Substantial fractions of thermolabile enzyme were produced as an immediate response

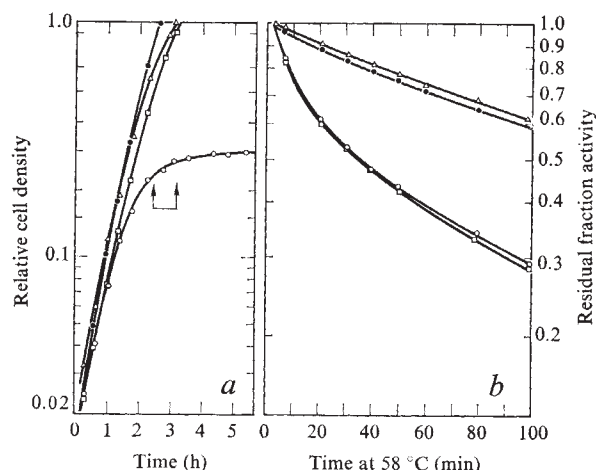


Fig. 3 Thermal sensitivity of the growth of treated cultures. Two freshly divided cultures of IH79, one with and one without streptomycin ($2 \mu\text{g ml}^{-1}$) were grown at 37°C for four generations in exponential growth medium. At time zero, each culture was diluted into two flasks of the same medium, one held at 37°C and the other at 42.5°C . During subsequent incubation with agitation at these temperatures the growth of the cultures was measured by reading the absorbance at 650 nm. At the time indicated by the first arrow (a), samples were removed and induced at 37°C . Induction was stopped at the time of the second arrow and the thermal degradation kinetics of the β -gal in each sample determined (b). Procedures and conditions were otherwise as described in the legend to Fig. 1. The strain IH79 normally grows well following a temperature upshift from 37°C to 43°C , but stops abruptly if shifted to 45°C . $\circ$, 42.5°C , + streptomycin ($2 \mu\text{g ml}^{-1}$); $\triangle$, 42.5°C , - streptomycin; $\square$, 37°C , + streptomycin ($2 \mu\text{g ml}^{-1}$); $\bullet$, 37°C , + streptomycin.

to streptomycin and limitations of permeability of the drug (revealed by pretreatment with EDTA-Tris¹³) played some role. The maximum immediate effect was, however, significantly less than the maximum late effect caused by streptomycin at $2 \mu\text{g ml}^{-1}$, and the high concentrations required ($\geq 30 \mu\text{g ml}^{-1}$) caused rapid cell killing. It therefore seems unlikely that the observed progressive effects of streptomycin resulted from changes in the intracellular concentration of active drug.

Mutations that confer resistance to high concentrations of streptomycin are mapped in the gene (*strA*) coding for a 30S subunit ribosomal protein (S12) and depress both the *in vitro* and *in vivo* error-producing effects of streptomycin⁹. Two resistant derivatives of IH79 and two independent resistant (*strA*) strains were found to have normal β -gal thermolability at concentrations of drug below $100 \mu\text{g ml}^{-1}$ but to produce a small amount of thermolabile enzyme (20% or less) when treated with $200 \mu\text{g ml}^{-1}$. We conclude that the *strA* locus mediates the production by streptomycin of the progressive effect.

The results reported here suggest that error feedback in gene expression exists in *E. coli*, and constitutes a basis for further investigation of the stability of information transfer in gene expression.

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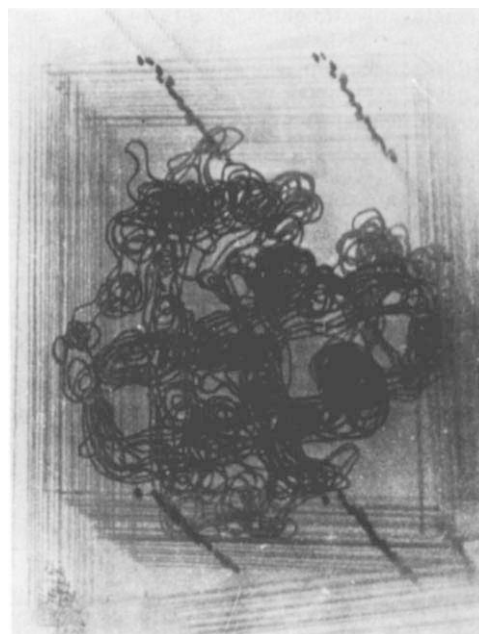
Structure of leghaemoglobin from lupin root nodules at 5 Å resolution

THE similarity in tertiary structure of mammalian haemoglobins¹⁻³ and myoglobins^{4,5} and of haemoglobins from invertebrates^{6,7} shown by X-ray analysis has been particularly important for our understanding of the biological evolution of these proteins. We have therefore studied the structure of leghaemoglobins—plant haemoglobins found in root nodules of leguminous plants—and compared the structures of seemingly unrelated, in terms of evolution, haemoglobins.

Leghaemoglobin from root nodules of the yellow lupin, *Lupinus luteus* L. is a monomeric haemoglobin of molecular weight about 16,000. The amino acid sequence for one component of leghaemoglobin from soya⁸ suggested a correlation in structural organisation of the leghaemoglobin and other haemoglobins. The physiological role of leghaemoglobin is concerned in some way with the fixation of atmospheric nitrogen, formed symbiotically in root nodules of a higher plant by the bacteria *Rhizobium*.

Leghaemoglobin was extracted from root nodules by chromatography on Al_2O_3 and DEAE cellulose (pH 7.0), the protein being eluted by the ammonium acetate buffer. Crystals reached a size of $0.5 \text{ mm} \times 0.5 \text{ mm} \times 1 \text{ mm}$ when grown in 28-33% saturated solutions of ammonium sulphate, buffered to pH 7.0 with 0.5 M ammonium acetate. The crystals are monoclinic ($a=92.95 \text{ \AA}$; $b=38.31 \text{ \AA}$; $c=52.15 \text{ \AA}$; $\gamma=98^\circ 50'$; space

Fig. 1 The three-dimensional Fourier synthesis of electron density of leghaemoglobin.



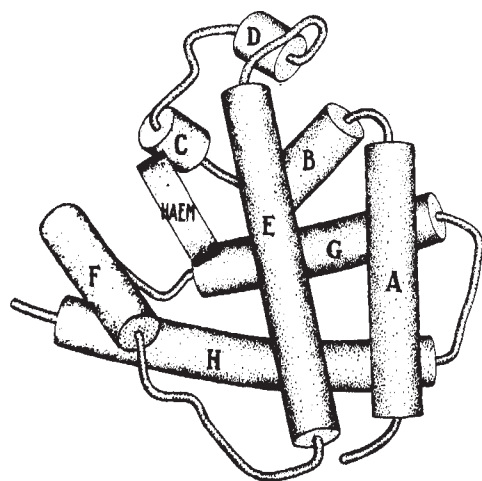


Fig. 2 The model of the leghaemoglobin molecule at 5 Å resolution.

group B2) and the unit cell contains four molecules, one per asymmetric unit⁹.

The structure was determined using four heavy atom derivatives containing HgI_4^{2-} , UO_2^{2+} , I_2 and 3-HgCl-pyridine, respectively. The heavy atom positions in HgI_4^{2-} and UO_2^{2+} derivatives were obtained by difference Patterson syntheses, the other two were resolved using difference Fourier syntheses and protein phases derived from HgI_4^{2-} and UO_2^{2+} derivatives. The heavy atom parameters, refined using a Muirhead program algorithm¹⁰, are listed in Table 1.

The best Fourier synthesis¹¹ of protein electron density at 5 Å resolution was calculated using 830 independent terms with the mean figure of merit 0.85. This revealed unambiguously the course of the polypeptide chain and the position of the haem group, with some indication of its orientation (Fig. 1). A very close resemblance between the tertiary structure of the leghaemoglobin and known animal haemoglobins and myoglobins was clearly shown. A similar myoglobin fold to that reported for sperm whale myoglobin^{4,5} was found and the electron density map clearly shows the eight straight 'α helices' A, B, ..., H (Kendrew's nomenclature¹²) joined by rather short irregular segments.

Estimation of the number of residues in α helices based on the lengths of straight segments of high density gives, for A, B, E and G helices in the leghaemoglobin, numbers close to those for sperm whale myoglobin (16, 16, 20 and 19 residues, respectively). The F helix seems to be longer (about 14 residues as against 9) and the H helix is slightly shorter (23

residues as against 26). Similar differences were observed in the haemoglobin from the marine annelid worm, *Glycera dibranchiata*¹³.

The haem group seems to be in the form of an oblate ellipsoid and can be distinguished by the highest value in the electron density map. As in other haemoglobins the haem position is between E and F helices with the narrow side facing the CD region. The haem plane is roughly normal to the line joining those parts of the E and F helices nearest to the haem.

The model of the leghaemoglobin molecule is shown in Fig. 2. The similarity in tertiary structure between leghaemoglobin and animal haemoglobins (myoglobins) seems to have some interesting implications for both evolutionary and functional aspects. If this similarity arises from a common origin of the animal and plant haemoglobins it would mean that haemoglobin was present in organisms which lived about 1.3×10^9 – 1.5×10^9 yr ago and in which the animal and plant kingdoms had their origin. The tertiary structures which have arisen during evolution therefore seem to have been conserved in widely divergent branches.

This similarity, however, parallels with the very poor correlation of the primary structures of haemoglobins (in all known haemoglobin sequences the true invariants are only 3 out of a total of 140–150 residues). It suggests that all the elements of three-dimensional structure which have traversed such variability in molecular chemistry unchanged, are indispensable not only for the stability of the molecular architecture but also for fulfilling their diverse functions.

We thank Professor M. F. Perutz, Drs H. Formanek, B. P. Atanasov and G. Ya. Zhiznevskaya for discussions, and Professor Ya. V. Peive who first drew our attention to the problems of leghaemoglobins.

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Table 1 Heavy-atom parameters

Compound	Site no.	Occupancy (electrons)	Fractional coordinates			B (Å ²)	R*
			x	y	z		
K_2HgI_4	1	56.9	0.017	0.081	0	110	0.57
	2	35.8	0.214	0.404	0.132	86	
	1	39.5	0.189	0.512	0.592	70	
	2	26.4	0.123	0.098	0.545	70	
$\text{UO}_2(\text{NO}_3)_2$	3	16.8	0.241	0.966	0.669	70	0.52
	4	15.9	0.145	0.079	0.707	70	
	1	31.0	0.117	0.029	0.116	70	
	2	29.4	0.159	0.133	0.155	70	
I_2							0.53
3-HgCl-pyridine	1	49.5	0.200	0.614	0.462	70	

$R^* = \sum (|F_{\text{PH}}| - |F_{\text{P}} + f_{\text{H}}|) / \sum (|F_{\text{PH}}| + |F_{\text{P}}|)$ calculated for all reflections, where F_{PH} is the structure factor for the derivative, F_{P} for the protein, and f_{H} for the heavy atoms.

Corrigendum

In the article "New pathway for metabolism of dopa" by M. H. O'Leary and R. L. Baughn (*Nature*, **253**, 52; 1975) the entries in the last column of Table 1 should read 0.09, 0.14, 0.13, 0.12 and 0.12 respectively.

obituary

Hugh Blackwell Evans, the Antarctic pioneer, died at the age of 100.

Hugh Evans was born at Alvington, Gloucestershire, in 1874 and died at Vermilion, Alberta on 5 February 1975. In 1896–7 he went in the *Edward* on a sealing expedition to Kerguelen, acting as assistant to Robert Hall, the ornithologist. In 1898–1900 he was a member of the *Southern Cross* Antarctic expedition under C. E. Borchgrevink, and was the last remaining member of the first ten men to winter on the Antarctic continent. In February 1900 he was one of the four men who achieved the "farthest south" on the Ross Ice Barrier. He was assistant to the zoologist, Nicolai Hanson, and when he died Evans took over his work, receiving the congratulations of Dr. Bowdler Sharpe. In 1901 he was offered a place on the staff of the National Antarctic Expedition, but being in Canada, farming, he was

unable to accept. He spent the remainder of his working life as a Prairie farmer. In the last couple of years he published three articles on his Antarctic experiences in the *Polar Record*.

Hyman Levy, the distinguished mathematician, died on February 27 at the age of 85.

Born in Edinburgh, Professor Levy was educated at the Universities of Edinburgh, Oxford and Gottingen. After four years on the staff of the National Physical Research Laboratory, he joined the Department of Mathematics at Imperial College, London, in 1920 as assistant professor, becoming professor in 1923 and head of the department in 1946 before retiring in 1954. The governing body of the college elected him one of its

members and appointed him Dean of the Royal College of Science (one of the constituent parts of Imperial College) in 1946, a post which he held for six years. In 1957, he was awarded a fellowship by the college. Levy's most significant contributions to mathematics, and the development of mathematical teaching, arose from his interests in numerical methods. This, and the related subject of statistics, remained his favourite field of mathematics throughout his life. He was also deeply interested in Marxism and was a member of a British delegation Communist Party which visited Russia in 1956. He investigated reports that Jewish writers, artists and intellectuals had been persecuted, and appalled by his findings, he wrote an exposure of such persecution in the Communist weekly *World News* in January, 1957. He later became very much disenchanted with Marxism.

announcements

Awards

J. E. Hyde and **S. E. Reynolds** have been awarded Harkness Fellowships of the Commonwealth Fund.

The Institute of Physics has made the following awards for various contributions: **Guthrie Medal and Prize** to **D. Tabor** (study of surfaces); **Glazebrook Medal and Prize** to **W. C. Marshall** (direction of the work of the Atomic Energy Authority, particularly the administration of Harwell); **Charles Chree Medal and Prize** to **R. Hide** (hydrodynamics of rotating fluids and their applications to the major planets); **Thomas Young Medal and Prize** to **D. J. Bradley** (laser physics, particularly the tunable dye laser); **Duddell Medal and Prize** to **E. Ruska** (electron microscopy and electron optics); **Charles Vernon Boys Prize** to **R. A. Stradling** (electronic properties of semiconducting and semimetallic solids, particularly quantum transport phenomena and magnetophonon spectroscopy); **Maxwell Medal and Prize** to **A. J. Leggett** (behaviour of condensed matter at very low temperatures, particularly the elucidation of the transition in liquid ^3He);

Bragg Medal and Prize to **W. A. Coates** (design and execution of demonstrations for lectures at the Royal Institution).

Sir Brian Flowers, FRS, has been awarded the **Chevalier de la Legion d'Honneur** for contributions to greater cooperation in the field of science between France and Great Britain.

R. M. Shackleton, FRS, has been awarded the **Clough Medal** for contributions to Scottish geology.

J. McManus has been awarded the **Clough Award** for work on modern sediments, particularly those of the Tay estuary.

Appointments

J. M. Hirst has been appointed Director of the Long Ashton Research Station, and to a chair and the Headship of the Department of Agriculture and Horticulture in the University of Bristol.

R. E. Richards will be the next Vice-Chancellor of the University of Oxford.

T. S. West has been appointed Director of the Macaulay Institute for Soil Research, Aberdeen.

R. Miledi, FRS, has been appointed Foulerton Research Professor by the Royal Society. Miledi is professor of biophysics at University College, London, and will continue his studies on nerve-muscle interaction there.

A Royal Society Research Professorship has been awarded to **J. W. Cornforth, CBE, FRS**. Cornforth is a director of the Milstead Laboratory of chemical enzymology at Shell Research and will take up his new appointment in the school of molecular sciences at the University of Sussex.

Miscellaneous

Developing Countries. The International Union of Pure and Applied Physics is to support the expenses of three scientists from developing countries during the Fifth International Conference on Atomic Masses and Fundamental Constants (AMCO-5), to be held in Paris from June 2–6, 1975. Applications (with curriculum vitae

and a written recommendation) to: AMCO-5 Secretariat, Institut d'Electronique Fondamentale, Bât. 220 Université Paris-Sud F-91405, Orsay, France.

Achievement Award. Applications are invited for this award, made annually for a British or Commonwealth achievement in the field of scientific instruments—research, design, or new technique—by the Worshipful Company of Scientific Instrument Makers. Applications (by March 31) to: The Clerk, Worshipful Company of Scientific Instrument Makers, Alliance House, 12 Caxton Street, London SW1, UK.

Essay Competition. The Bertrand Russell Memorial Logic Conference is offering a prize of £200 for an essay that examines in detail some aspect of the relationship between mathematics and the development of social or economic conditions. To be of general interest to mathematicians and include a consideration of current mathematical practice. Deadline: February 1, 1976. Further details from: Dr A. Slomson, School of Mathematics, The University, Leeds LS2 9JT, UK.

Foulkes Foundation. Offers a number of fellowships to science or other suitable graduates who have recently received a Ph.D. or equivalent, and who wish to take a full second degree course in medicine. Also for recently qualified medical men and women wishing to take a scientific, technological or other suitable subject. Applications to: D. W. FitzSimons, Secretary, Foulkes Foundation Fellowship, The CIBA Foundation, 41 Portland Place, London W1N 4BN, UK.

International meeting

April 15–16, **Understanding Energy Systems**, London (R. A. Davies, Operational Research Society, Sixth Floor, Neville House, Waterloo Street, Birmingham B2 5TX, UK).

Reports and publications

Great Britain

Theoretical Chemistry: Past and Future. By Professor C. A. Coulson. (An Inaugural Lecture delivered before the University of Oxford on 13 February 1973.) Pp. 38. (Oxford: Clarendon Press, London: Oxford University Press, 1974.) 50p net. [211]
University College of Wales, Aberystwyth. Report of the Welsh Plant Breeding Station for 1973. Pp. 133. (Plas Gogerddan, Near Aberystwyth: Welsh Plant Breeding Station, 1974.) 50p. [211]
Conservation in Museums and Galleries: a Survey of Facilities in the United Kingdom. Pp. 115. (London: United Kingdom Group, International Institute for Conservation of Historic and Artistic Works, 608 Grand Buildings, Trafalgar Square, 1974.) £1. [221]
Philosophical Transactions of the Royal Society of London. A: Mathematical and Physical Sciences. Vol. 277, No. 1270: A Discussion on the Origin of the Cosmic Radiation. Organized by G. D. Rochester, and A. W. Wolfendale. Pp. 317–501 + plates 12 and 13. UK £6.85; Overseas £7.05. Vol. 277, No. 1271: The Theory, Manufacture, Structure and Performance of N. P. I. X-ray Gratings. By A. Franks, K. Lindsey, J. M. Bennett, R. J. Speer, D. Turner, and D. J. Hunt. Pp. 503–543 + plates 16–19. UK £1.90; Overseas £2. (London: The Royal Society, 1975.) [231]

Person to Person

Weinberg's Differential Rule. Additional data required to test this rule. Would workers routinely ascertaining and blood-typing twins at birth please let me have the following data: among the pairs known to be DZ on basis of blood groups alone, how many are same-sexed, and how many are opposite-sexed? (Dr W. H. James, Galton Laboratory, Department of Human Genetics and Biometry, University College, London NW1 2HE, UK).

Enzymes. A summer course is being organised by the Massachusetts Institute of Technology on 'Enzymes and their Use in Analysis and Clinical Diagnosis', from June 23–27. Aims to develop ability to use enzymes as analytical reagents and measure their activities. Director of the Summer Session, Room E19-356, MIT, Cambridge, Massachusetts 02139).

Echange de Maisons. Famille à Londres veut faire un échange de maisons avec famille française, août 9–16. Offert: maison de 4/5 chambres, 4 étages, tout ce qu'il faut pour bébés/enfants; location paisible dans un faubourg agréable, près de Greenwich, 20 minutes par le train au centre de Londres; visites intéressantes aux environs. Demandé: maison, ville ou campagne, endroit agréable, Bretagne, Paris ou le nordouest, pour famille de 3 enfants (D. Davies, 32 St Margaret's Passage, London, S.E.13, UK).

Mitochondrial Structure and Function. This course, sponsored by the University Board of Studies in Biochemistry, will be held from April 7–10 and is designed for post-graduate or other qualified students in the London area. Consists of a series of lectures and practical demonstrations on mitochondrial bioenergetics. Registration fee: £10 (Dr P. J. Quinn, Department of Biochemistry, Chelsea College, Manresa Road, London SW3 6LX, UK).

House Exchange. House sought in English or Scottish fishing village, fortnight August, preferably near the spring tides. Offer in exchange our house in Stratford-upon-Avon. Family of two adults, two boys (both primary age), (R. C. Hardwick, 3 Mayfield Avenue, Stratford-upon-Avon, Warwickshire, CV37 6XB, UK).

There will be no charge for this service. Send items (not more than 60 words) to Robert Vickers at the London office. The section will include exchanges of accommodation, personal announcements, and scientific queries. We reserve the right to decline material submitted. No commercial transactions.

Other countries

Annals of the South African Museum. Vol. XLVII, Part 5: Contributions to the Knowledge of South African Marine Mollusca. Part VII. Revised Fauna List. By K. H. Barnard. Pp. 663–781. R.10.40. Vol. XLVII, Part 6: Contributions to the Knowledge of South African Marine Mollusca. Index: Parts I–LVII. Compiled by B. Kensley. Pp. 783–825. R.3. (Cape Town: South African Museum, 1974.) [3012]

Brain Structures at Work in Organism-Environment Interactions. By Edward Friel. Pp. 41. (Seattle, Washington: Edward Friel, 1837–12th Avenue West, 1974.) [3112]

Smithsonian Contributions to Zoology. No. 184: The Genus *Coptocarpus* Chaudoir of the Australian Region with Notes on Related African Species (Coleoptera: Carabidae: Oodini). By Terry L. Erwin. Pp. iii + 25. (Washington, DC: Smithsonian Institution Press, 1974. For sale by US Government Printing Office.) 85 cents. [3112]

Cahiers de Micropaléontologie. No. 3. Par A. Rouvillois. Pp. 19 + 4 planches. (Paris: Centre National de la Recherche Scientifique, 1974.) 15 francs. [3112]

The Discrimination of Birthtypes in Relation to Disease. By John M. Addey. Pp. 26. (Green Bay, Wisconsin: The Cambridge Circle Press, 463 Vande Hei Road, 1974.) [31]

Australia: Commonwealth Scientific and Industrial Research Organisation. Division of Soils Technical Paper No. 24: INTERPS—a Computer Program for Estimating Chemical Analyses from Light Intensity Measurements Recorded with Colorimetric Methods of Analysis. By J. D. Colwell. Pp. 15. (Melbourne: CSIRO, 1974.) [31]

European Organisation for Nuclear Research—CERN 74-24: PL-11—a Programming Language for the DEC PDP-11 Computer. By Robert D. Russell. Edited by T. C. Streater. Pp. xov + 104. (Geneva: CERN, 1974.) [61]

Comptes Rendus des Travaux du Laboratoire Carlsberg. Vol. 40, No. 1: Microsynchronised Studies on the Macromolecules of Heat Synchronised *Tetrahymena pyriformis* GL. By Jytte R. Nilsson and Erik Zeuthen. Pp. 1–18 + 15 plates. Vol. 40, No. 2: Assessment of an Industrial Homogenizer for Protein and Enzyme Solubilization from Spent Brewery Yeast. By D. A. Whitworth. Pp. 19–32. Vol. 40, No. 3: Effects of Nini-ionic Surfactants on Alkane Utilization by Yeast-Structure-Action Relationships. By D. A. Whitworth. Pp. 33–46. Vol. 40, No. 4: Conversion of Hexadecan-1-OL by Extracts of *Candida lipolytica*. By D. A. Whitworth. Pp. 49–56. Vol. 40, No. 5: A Method for Synchronization of Cell Division and Macromolecule Formation in the Ciliate *Tetrahymena vorax*. By Howard E. Buhse, Jr., and Leif Rasmussen. Pp. 57–68. Vol. 40, No. 6: Respiration and Macromolecular Synthesis During Cell Division and Macromolecule Formation in Heat Synchronised *Tetrahymena vorax*. Strain V. By Howard E. Buhse, Jr., Kristen Hamburger and Anne Lykkesfeldt. Pp. 69–76. Vol. 40, No. 7: Induced Macromolecule Formation in *Tetrahymena vorax* Strain V₂ Patterns of Respiration. By Howard E. Buhse, Jr., and Kristen Hamburger. Pp. 77–90. (Copenhagen: Danish Science Press, Ltd., 1974.) Dkr. 11.50 each. [81]

United States Department of the Interior: Fish and Wildlife Service. Togiak National Wildlife Refuge, Alaska—a Proposal. Pp. 16. Selawik National Wildlife Refuge, Alaska—a Proposal. Pp. 12. Noatak National Arctic Range, Alaska—a Proposal. Pp. 16. Koyukuk National Wildlife Refuge, Alaska—a Proposal. Pp. 16. Iliamna Alaska National Resource Ranga—a Proposal. Pp. 24. Yukon Flats National Wildlife Refuge, Alaska—a Proposal. Pp. 24. Yukon Delta National Wildlife Refuge, Alaska—a Proposal. Pp. 24. Alaska Coastal National Wildlife Refuge—a Proposal. Pp. 32. Arctic National Wildlife Refuge—a Proposal. Pp. 24. Proposed Alaska National Wildlife Refuges. Pp. 8. (Washington, DC: US Department of the Interior, 1974.) [81]

Soil Conservation: a Guide to Farming Practices in the Sugarcane Industry. (Bulletin No. 20.) Pp. 27. (P.O. Mount Edgecombe, S. Africa: The Experiment Station of the South African Sugar Association, 1974.) [91]

World Health Organization. Monograph Series No. 61: Handbook on Human Nutritional Requirements. By R. Passmore, B. M. Nicol, M. Narayana Rao, in collaboration with G. H. Beaton and E. M. Demayer. Pp. vii + 66. Sw.fr.12. Offset Publication No. 7: Health Education—a Programme Review. (A Report by the Director-General of the World Health Organization to the Fifty-third Session of the Executive Board.) Pp. 78. Sw.fr.17. Offset Publication No. 8: The Current and Future Use of Registers in Health Information Systems. By Eileen M. Brooke. Pp. ii + 43. Sw.fr.14. (Geneva: WHO; London: HMSO, 1974.) [91]

World Health Organization. Public Health Papers, No. 57: The Teaching of Human Sexuality in Schools for Health Professionals. Edited by Dr. D. R. Mace, Dr. R. H. O. Bannerman and Dr. J. Burton. Pp. 47. (Geneva: WHO; London: HMSO, 1974.) Sw.fr.5. [101]

Australia: Commonwealth Scientific and Industrial Research. Annual Report of the Division of Irrigation Research, 1973/1974. Pp. iv + 66. (Griffith, NSW: CSIRO, 1974.) [101]

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World Health Organization. Technical Report Series No. 557: Evaluation of Certain Food Additives: Eighteenth Report of the Joint FAO/WHO Expert Committee on Food Additives. Pp. 37. (Geneva: WHO; London: HMSO, 1974.) Sw.fr.5. [131]